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8) ABSTRACT

The present investigations show the feasibility of analyzing the cerebral metabolism of amino acids and related metabolites by ^{13}C-and ^{14}C-labelling using labelled acetate and glucose as markers for glial and neuronal metabolism, respectively. Using [^{13}C]acetate, it was shown that glial cells export ~60% of their TCA cycle intermediates, mostly as glutamine, and that this glutamine is used by neurons partly as an energy reserve, partly it is converted directly to glutamate and GABA. Using [^{13}C]glucose, the glial process of pyruvate carboxylation was shown to compensate fully for the loss of glutamine. The mechanism of action of two neurotoxins, fluorocitrate and 3-nitropropionate, was elucidated. The latter toxin was shown to inhibit the TCA cycle of GABAergic neurons selectively. Formation of pyruvate and lactate from glial TCA cycle intermediates was demonstrated *in vivo*. This pathway may be important for the glial inactivation of transmitter glutamate and GABA. The results illustrate glia-neuronal interactions, and they suggest the applicability of ^{13}CNMR spectroscopy to the detailed study of the cerebral metabolism of amino acids in the intact, unanesthetized human brain.

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PREFACE

The present work was carried out at the Norwegian Defence Research Establishment (NDRE), Division for Toxicology, 1990 - 1994. I wish to thank my supervisor and friend professor Frode Fonnum for creating an inspiring scientific environment, for his support and advice. I also wish to thank the Directors of the NDRE, drs. Erik Klippenberg and Nils Holme, for providing excellent research opportunities. The interest and enthusiasm shown by Director Holme has been an encouragement during the years at NDRE. I am also very grateful to my colleagues at NDRE. I thank my room mate and good friend dr. Ragnhild Elisabeth Paulsen for inspiring discussions and help, and drs. Else Marie Fykse, Svein Roseth, Sigrun Sterri, Pål Aas, Arne Bøyum, Per Kristian Opstad, Knut Kristian Skrede and Pål Wiik for in various ways taking the time to help me with practical problems as well as for their providing a sound, critical and inspiring milieu. I further wish to thank Evy Grini Iversen, Arnt Johnsen, Rita Tansø, Ann Helen Haugen, Hanne Wennemo and Hermann Arvesen for collaboration and help.

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I respectfully acknowledge the contribution of previous researchers in the field, particularly those whose work formed the basis of the «two-compartment» model of the cerebral metabolism, on which the present work is based: Harry Waelsch, Soll Berl, Donald D. Clarke, Dominick P. Purpura, Cees S. Van den Berg, Robert Balázs, Jill E. Cremer, Abel Lajtha and Micky Gaitonde.

Lastly, I thank my dear wife Mette for patience and support. The present work is, in a true sense, the result of our daily collaboration.

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CONTENTS

LIST OF PUBLICATIONS

I. Hassel B., Paulsen R.E., Johnsen A.J., and Fonnum F. (1992) Selective inhibition of astrocytes *in vivo* by fluorocitrate. *Brain Res.* **576,** 120-124.

II. Hassel B., Sonnewald U., Unsgård G., and Fonnum F. (1994) NMR spectroscopy of cultured astrocytes. Effects of glutamine and the gliotoxin fluorocitrate. *J. Neurochem.* **62,** 2187-2194.

III. Hassel B., Sonnewald U., and Fonnum F. (1995a) Glial-neuronal interactions as studied by cerebral metabolism of [2-^{13}C]acetate and [1-^{13}C]glucose. An *ex vivo* ^{13}C NMR spectroscopic study. *J. Neurochem.* **64,** 2773-2782.

IV. Hassel B. and Sonnewald U. Glial formation of pyruvate and lactate from TCA cycle intermediates. Implications for the inactivation of transmitter amino acids? *J. Neurochem.* **65,** 2227-2234.

V. Hassel B. and Sonnewald U. (1995b) Selective inhibition of the TCA cycle of GABAergic neurons with 3-nitropropionic acid *in vivo*. *J. Neurochem* **65,** 1184-1191.

VI. Hassel B., Westergaard N., Schousboe A., and Fonnum F. (1995c) Metabolic differences between primary cultures of astrocytes and neurons from cerebellum and cerebral cortex. Effecs of fluorocitrate. *Neurochem. Res.* **20,** 343-350.

AIMS OF THE STUDIES

1. To establish or verify methods for the identification of glial and neuronal amino acid metabolism separately, and to use these methods to study glial-neuronal interactions (Papers I, III, IV).

2. To explore the possibilities of ^{13}C NMR spectroscopy in elucidating brain metabolism as determined from the labelling of amino acids and related metabolites from ^{13}C-labelled precursors (Papers II, III, IV, V).

3. To elucidate the mechanisms of action of two neurotoxins which are used as tools in neurobiology and which are inhibitors of energy metabolism, fluorocitrate and 3-nitropropionic acid (Papers I, II, V, VI).

4. To study the metabolic heterogeneity of brain cells with respect to amino acid metabolism by determining the content, formation and release of amino acids and related metabolites from primary cultures of astrocytes and neurons from two different parts of the brain, the prefrontal cortex and the cerebellum (Paper VI).

THE CEREBRAL METABOLISM OF AMINO ACIDS AND RELATED METABOLITES AS STUDIED BY ^{13}C AND ^{14}C LABELLING

1 INTRODUCTION

There is a need in basic and clinical neurobiology for techniques that allow the study of the cerebral metabolism in the unanestetized, living brain. Metabolic studies of the human brain have largely been confined to analyses of tissue obtained at autopsy. Studies of diseases of the central nervous system which are performed on material obtained post mortem have to take into account the effects of medication, concurrent illnesses and the agonal state. Further, the changes seen in such material often represent the end stage of the disease at which time the primary events of the disease may be overshadowed by secondary changes. NMR spectroscopy may in the near future offer the possibility to study the metabolism of the intact, unanesthetized human brain in a non-invasive manner without the use of radioactive isotopes, which allows continuous monitoring or frequent follow up studies without the hazards of radiation.

The brain displays an extreme cellular heterogeneity. It is customary to assume that the human brain consists of approximately 10^{11} nerve cells that can be subdivided into 1,000 different cell types (e.g. Jessel and Kandel, 1993). In addition to neurons the main categories of cells are the glia, the ependymal cells, the epithelium of the choroid plexus, and the cells of the vascular walls. Even the blood cells of the cerebral blood volume may be seen as contributing to the cellular complexity of the living brain. Obviously, the brain is also metabolically very heterogeneous. For instance the synthesis and inactivation of the various neurotransmitters require specific metabolic machineries. Further, the metabolic rate of the individual cells changes with time both since cellular activity varies with the brain's responses to a changing external environment and since the brain is subjected to various intrinsic biological rythms.

Against this intricate backdrop limiting the study of brain metabolism to the formation of glutamate, GABA, aspartate, glutamine and alanine may appear as an oversimplification. These amino acids are, however, of great importance in the cerebral energy metabolism. Furthermore, glutamate and GABA are considered to be the quantitatively most important excitatory and inhibitory cerebral neurotransmitters, respectively. Therefore establishing methods for the visualization of the metabolism of these amino acids, using techniques which may be applied to the living human brain, may be useful both for basic and clinical neurobiolgy.

1.1 The concept of two metabolically separate TCA cycles in the brain

In the 1960's the discovery was made that administration of ^{14}C-labelled fatty acids (acetate, propionate, butyrate), bicarbonate, glutamate and aspartate to experimental animals led to a higher incorporation of radioactivity into brain glutamine than into glutamate (Berl et al., 1961; 1962; Waelsch et al., 1964; O'Neal and Koeppe, 1966; O'Neal et al., 1966). This was surprising since glutamate is the immediate precursor for glutamine, and since the cerebral pool of glutamate is larger than the pool of glutamine, which is why one would expect glutamate to be more highly labelled than glutamine, as seen after administration of ^{14}C-labelled glucose, lactate and pyruvate (Cremer, 1964; Gaitonde, 1965; O'Neal and Koeppe, 1966; Van den Berg et al., 1969; Shank and Campbell, 1984a). The paradox was resolved by assuming the presence of two pools of glutamate in the brain, one large pool which is rapidly labelled by [^{14}C]glucose, but not by [^{14}C]acetate, and which is not metabolized to glutamine, and one small pool which is labelled by [^{14}C]acetate etc., and which is the precursor for glutamine (Figure 1.1). The model implied that the two pools of glutamate were associated with two different TCA cycles representing two separate metabolic compartments, the «large» and «small» compartment, corresponding to the large and the small pool of glutamate, respectively. The TCA cycle of the large compartment was termed the «energy cycle» (Van den Berg et al., 1969; Van den Berg, 1973) since it was shown that the intermediates of this cycle were not lost, but were passed repeatedly through the cycle to yield CO_2, H_2O and therefore ATP. (The glutamate derived from the 2-oxoglutarate of this cycle was assumed to be quantitatively converted back to 2-oxoglutarate without any loss of intermediates from the TCA cycle.) The TCA cycle of the small compartment, on the other hand, was termed the «synthetic» cycle» since it was involved in the synthesis of glutamine, a process which implies that 2-oxoglutarate is continuously lost from the cycle (Van den Berg et al., 1969; Van den Berg, 1973).

The continuous loss of 2-oxoglutarate from the «synthetic» TCA cycle would necessitate a compensatory process to replenish the TCA cycle with intermediates. This could either be by some anaplerotic process by which TCA cycle intermediates were produced in the small compartment, or it could be by entry of TCA cycle intermediates (or their amino acid derivatives) into the small compartment. It was suggested at an early stage that formation of glutamine was linked to the anaplerotic process of pyruvate carboxylation (Waelsch et al. 1964) by which oxaloacetate, a TCA cycle intermediate, is made from pyruvate, a non-TCA cycle intermediate. Pyruvate carboxylation was found to take place in the brain, and was assessed to account for approximately 10 % of the cerebral consumption of glucose (Waelsch et al., 1964; O'Neal and Koeppe, 1966; Van den Berg, 1973). TCA cycle intermediates (or their amino acid derivatives) could in principle enter the small compartment from three different sources; from the circulation, from the breakdown of protein, or from the large compartment with its «energy» TCA cycle. Of three alternatives the latter has received most attention.

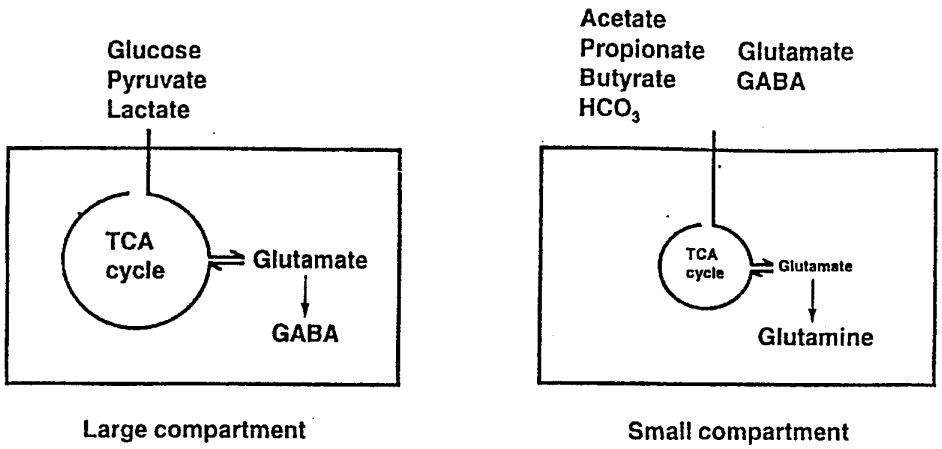

Figure 1.1 A schematic representation of the two metabolic compartments in brain: the large compartment with the «energy TCA cycle» and the large pool of glutamate, and the small compartment with its «synthetic TCA cycle» and the small pool of glutamate which serves as precursor for glutamine.

Attempts were made to relate the two cerebral metabolic compartments to cellular structures. On the basis of neurochemical and anatomical data Balázs et al. (1970) suggested that the small compartment corresponded to glia. It was, however, not until the immunohistochemical localization of glutamine synthetase in glia (Martinez-Hernandez et al., 1977; Norenberg and Martinez-Hernandez, 1979; Patel, 1982), that the small compartment with its synthetic TCA cycle could be conclusively ascribed to glia. The main anaplerotic enzyme in brain, pyruvate carboxylase (EC 6.4.1.1) was also shown to be active in cultured glia and not in neurons (Yu et al., 1983; Shank et al.,1985; Kaufman and Driscoll, 1992). This was consistent with the proposition made by Waelsch et al. (1964) that pyruvate carboxylation is a prerequisite for glutamine formation, and confirmed the idea of the synthetic TCA cycle being localized in glia.

[^{14}C]Glucose was found to yield high labelling of GABA. Since glutamate decarboxylase, the enzyme which catalyzes the formation of GABA, was known to have a neuronal localization, and since GABA had been identified as a neurotransmitter (Fonnum et al., 1970; for review see Fonnum, 1987), the glucose-metabolizing compartment and hence the "energy TCA cycle" was ascribed to neurons (for review, see Balázs et al., 1973a,b). A subdivision of the large compartment was necessary, however, since it was shown that the pool of glutamate which served as precursor for GABA, in fact, had to be quite small (Balázs et al., 1973b), a conclusion which has been confirmed in later studies (Cremer, 1978; Ottersen and Storm-Mathisen, 1985; Ottersen, 1989; Fonnum, 1991).

1.2 Interchange of metabolites between glia and neurons. The flux from glia to neurons.

The model of two cerebral TCA cycles was developed to describe an exchange of intermediates between the cycles, i.e. a trafficking of metabolites between neurons and glia (Figure 1.2). The labelling of GABA from [^{14}C]acetate was thought to represent the transfer of glutamine, labelled from [^{14}C]acetate, to GABAergic neurons, and its subsequent conversion to glutamate and hence GABA (Van den Berg et al., 1969; Van den Berg, 1973). Other studies suggested transfer of glutamine to the large pool of glutamate (Nicklas and Clarke, 1969; Van den Berg and Van den Velden, 1970). Later, glutamine was shown to be a good precursor for the releasable pool of both GABA (Tapia and Gonzalez, 1978) and glutamate (Cotman and Hamberger, 1978) *in vitro*. The latter study also showed that [^{14}C]glutamine is a better precursor for releasable glutamate in cerebral slices than is [^{14}C]glucose. Also when radiolabelled glucose and glutamine were injected intracerebroventricularly *in vivo*, glutamine was the better precursor for releasable glutamate and GABA in brain slices (Ward et al., 1983). Further, Bradford et al. (1978) showed that glutamine is an excellent *in vitro* metabolic substrate for isolated nerve terminals, a finding which was confirmed by Yudkoff et al. (1989). *In vitro* Sonnewald et al. (1993a) recently showed transfer of astrocytic glutamine labelled from [2-^{13}C]acetate to GABAergic neurons with subsequent ^{13}C labelling of GABA.

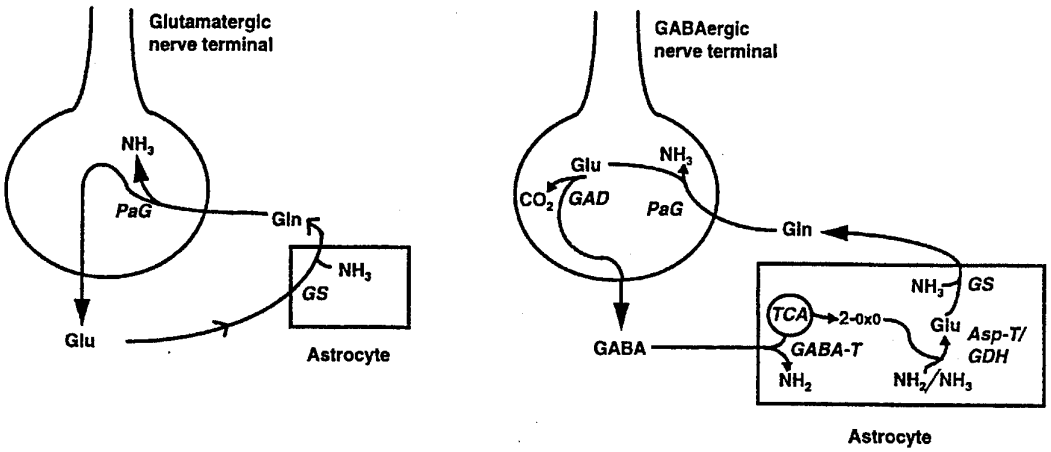

Figure 1.2 The «glutamine cycle». A glutamatergic nerve terminal (left) receives astrocytic glutamine and releases glutamate which in turn is taken up by astrocytes and converted to glutamine. A GABAergic nerve terminal (right) receives astrocytic glutamine, converts it to glutamate and hence to GABA. GABA is released into the synaptic cleft, and is taken up by astrocytes, metabolized through the astrocytic TCA cycle, and converted to glutamine. ASP-T: *aspartate transaminase: transaminates 2-oxoglutarate to glutamate;* GABA-T: *GABA transaminase: transaminates GABA to succinic semialdehyde;* GAD: *glutamic acid decarboxylase: decarboxylates glutamate to GABA;* GDH: *glutamate dehydrogenase: converts 2-oxoglutarate to glutamate by reductive amination;* GS: *glutamine synthetase: amidates glutamate to glutamine;* PaG: *phosphate-activated glutaminase: deamidates glutamine to glutamate. Gln: glutamine; Glu: glutamate; 2-oxo: 2-oxoglutarate; TCA: tricarboxylic acid cycle.*

1.3 Transfer of glutamate and GABA from neurons to glia

Several studies, both *in vivo* and *in vitro*, showed conversion of ^{14}C-labelled glutamate and GABA to glutamine, a sign that these amino acids to some extent were metabolized in the small compartment, i.e. glia (Berl et al., 1961; Berl, 1965; Berl and Purpura, 1966; Balázs et al., 1970; Machiyama et al., 1970). Since GABA and glutamate were suggested to be neurotransmitters (For reviews, see Krnjevic 1970; Fonnum, 1984; 1987), this indicated that glial uptake mechanisms could be involved in the inactivation of transmitter amino acids (e.g. Henn et al., 1974). The concept of a glutamine-glutamate and a glutamine-GABA cycle ("the glutamine cycle", for short) developed, implying that glutamate or GABA released from neurons during transmission, are taken up by glia and converted to glutamine. Glutamine, in turn, is transferred back to neurons as a precursor for glutamate and GABA (Figure 1.2) (Balázs et al., 1970; Van den Berg, 1973; Hertz, 1979; Duce and Keen 1983; Yudkoff et al., 1988; Paulsen et al., 1988). Glial uptake of transmitter amino acids was recently substantiated with the cloning and immunohistochemical detection of a GABA transporter present both in glia and neurons (Guastella et al., 1990; Radian et al.,

1990) and a glutamate transporter, GLT-1, present in glia (Pines et al., 1992; Danbolt et al., 1992; Levy et al., 1993). Two other glutamate transporters are known: GLAST, which is present both in glia and neurons, and EAAC1, which has a neuronal distribution (Rothstein et al., 1994). In favour of the concept of a glutamine cycle was also the finding that astrocytic processes in the vicinity of glutamatergic synapses contained glutamine synthetase (Derouiche and Frotcher, 1991). Participation of glia in the termination of glutamatergic neurotransmission was recently demonstrated electrophysiologically *in vitro* (Mennerick and Zorumski, 1994).

The relative importance of the glial vs. the neuronal uptake of transmitter amino acids *in vivo* has not been determined, however. Autoradiographic studies showed avid uptake of [^{3}H]GABA into nerve terminals in cortical slices (Iversen and Johnston, 1971; Iversen and Bloom, 1972). Uptake of glutamate into glutamatergic terminals was described by Divac et al. (1977). More recently Gundersen et al. (1993), using anti-D-aspartate antibodies, showed a greater uptake of exogeneous D-aspartate, a substrate for the glutamate transporter in the plasma membrane, into nerve terminals than into glia in brain slices. The classical neurochemical studies on compartmentation, on which the concept of the glutamine cycle was subsequently based, were designed to demonstrate metabolic compartmentation; they were not designed to elucidate a glutamine cycle. Methodologically, they therefore contain possible sources of error when interpreted in favour of glutamine cycling. For instance, in some *in vivo* experiments [^{14}C]glutamate was administered to the neocortical surface (Berl, 1965), or it was injected into the cisterna magna (Berl et al., 1961; Berl and Purpura, 1966), from where the label would first reach the surface of the cerebellum, brain stem and cerebral cortex. In these studies glutamine attained a higher specific activity than glutamate, indicating glial metabolism of glutamate. This mode of administration may, however, favour glial uptake of label. In neonatal kitten, in which glia is not fully developed, [^{14}C]glutamate applied to the surface of the brain gave a higher specific activity of glutamate than of glutamine (Berl, 1965; Berl and Purpura, 1966), indicating neuronal metabolism of glutamate. Another factor which may favour labelling of glutamine over glutamate, is the use of C-1-labelled glutamate (Machiyama et al., 1970) and GABA (cf. Balázs et al., 1970). When glutamate is labelled in the C-1 position, its uptake into neurons will lead to its repeated passage through the TCA cycle with rapid loss of label as CO_2. In glia, on the other hand, C-1-labelled glutamate may be converted directly to glutamine by glutamine synthetase without loss of label, which results in better labelling of glutamine than of glutamate. Glial conversion of GABA to glutamine also involves metabolism through the TCA cycle with loss of label as CO_2. As shown in Paper III, however, a large fraction of the glial TCA cycle intermediates are converted to glutamine which will retain much of the label (cf. Figure 1.2).

In conclusion, studies with ^{14}C-labelled glutamate and GABA show glial uptake and metabolism of these amino acids, but they do not necessarily support the idea that transmitter glutamate and GABA are taken up by glia during normal neuronal activity, nor do they necessarily imply that glial uptake of these amino acids is quantitatively more

important than the neuronal uptake. It seems reasonable that reuptake of transmitter amino acids into the nerve terminals by high affinity uptake (Divac et al., 1977) is quantitatively more important than the glial uptake because of the proximity of the nerve terminal to the released transmitter. This discussion is related to whether uptake of transmitter glutamate and GABA is involved in the termination of the postsynaptic response effected by the transmitter. For instance, termination of the glutamate-induced postsynaptic response mediated by the activation of AMPA receptors appears to be by dissociation of glutamate from the receptor (Clements et al., 1992). Dissociation of glutamate from its receptor depends on a concentration gradient which may be maintained by uptake mechanisms. Termination of the NMDA receptor response, on the other hand, is probably caused by desensitization of the receptor rather than by dissociation (Lester et al., 1990; Hestrin et al., 1990). Termination of the GABA-A receptor response is also caused by desensitization of the receptor (e.g. Leviatan et al., 1988).

2 DISCUSSION OF METHODS

2.1 Labelled acetate and glucose as markers for glial and neuronal metabolism

Glucose is the principal metabolic fuel for the brain. The arteriovenous difference for glucose correlates strongly with oxygen consumption and CO_2 formation (Gibbs et al., 1942; McIlwain and Bachelard, 1985), and reduction in available glucose, as in insulin-induced hypoglycemia, is associated with a reduction in cerebral oxygen consumption and loss of brain function. Other endogenous substrates do not readily substitute for glucose in acute hypoglycemia, although it was recently shown that administration of lactate may protect or restore neuronal function both in hypoglycemic humans (Maran et al., 1994) and in cerebral slices (Izumi et al., 1994).

As noted previously, [^{14}C]glucose labels the large pool of glutamate (Cremer 1964; Gaitonde, 1965; O'Neal and Koeppe, 1966; Van den Berg et al., 1969; Balázs et al., 1970) which corresponds to the neuronal compartment, whereas [^{14}C]acetate labels the small pool of glutamate (O'Neal and Koeppe, 1966; O'Neal et al., 1966; Van den Berg et al., 1966, 1969; Berl and Frigyesi, 1968; 1969) which corresponds to the glial compartment. An index of the compartment in which a labelled substrate is metabolized, is given by the specific activity of glutamine relative to that of glutamate, «the relative specific activity (RSA) of glutamine». This ratio becomes less than 1 with [^{14}C]glucose and greater than 1 with [^{14}C]acetate.

By autoradiography radiolabelled glucose was shown to preferentially enter neurons (Minchin and Beart, 1975), whereas acetate was shown to enter glial cells (Minchin and Beart, 1975; Muir et al., 1986). Sonnewald et al. (1993a) showed in cultured cortical neurons, which are mainly GABAergic (Yu et al., 1984), that [2-^{13}C]acetate failed to label

GABA. In cultured astrocytes, on the other hand, [2-^{13}C]acetate strongly labelled glutamine. As noted by Clarke (1991), however, the exclusively glial localization of acetate thiokinase, the initial enzyme in the metabolism of acetate, remains to be demonstrated. Cerdan et al. (1990) calculated that neurons derived 5 % of their acetyl-CoA from acetate, whereas glia derived 48 % of their acetyl-CoA from acetate, as determined ^{13}C NMR spectroscopy of brain extracts after *in vivo* infusion of [1,2-^{13}C2]acetate. This study has been criticized for not subtracting the natural abundance of 13C, which may have influenced the results (McLean et al., 1993; Bachelard and Badar-Goffer, 1993). Even so, it should be kept in mind that the labelling of amino acids from acetate may not be entirely due to glial metabolism.

It is an important presupposition in the present work that glutamine is a precursor for neuronal glutamate and GABA *in vivo*. As pointed out by Fonnum (1991), however, exogenous glutamine which is injected into brain tissue *in vivo*, has not been shown to be a good precursor for releasable glutamate and GABA. Administration of labelled acetate and glucose to brain tissue as in the present work, leads to labelling of endogenous amino acids. This is in contrast to the use of labelled amino acids, which, strictly speaking, only gives information on the cerebral handling of exogenous amino acids.

In the present papers no distinction is made between the different types of glial cells with respect to amino acid metabolism, the reason being that glutamine synthetase is present in glia both in grey and white matter, which could represent both astrocytes and oligodendrocytes (Martinez-Hernanez et al., 1977).

[2-^{13}C]Acetate and [1-^{13}C]glucose both yield [2-^{13}C]acetyl-CoA (Papers II-V), which makes the comparison of the two substrates as precursors for amino acids more meaningful than when for instance [1-^{14}C]acetate and [U-^{14}C]glucose are used (Paper I). With [2-^{13}C]acetate and [1-^{13}C]glucose, glutamate and glutamine initially become labelled in the C-4 positions, and GABA is labelled in the corresponding C-2 position. When the label is passed through the TCA cycle, the C-2 and C-3 positions in glutamate, glutamine (and in aspartate) are labelled next, together with the C-3 and C-4 of GABA. The importance of this lies in the short relaxation times of these carbon positions in ^{13}C NMR spectroscopy (see below) as compared to the C-1 and C-5 positions of glutamate and glutamine, the C-1 and C-4 of aspartate and the C-1 of GABA, which are all carboxyl groups. These groups are labelled from [1-^{13}C]acetate and [2-^{13}C]glucose (cf. Van den Berg et al., 1969; Badar-Goffer et al., 1990). The labelling of the C-2, C-3 and C-4 positions of glutamate, glutamine and GABA offers the possibility of calculating the enrichment ratios between the various position (Papers II, III and V) which may be used as indices of various metabolic fluxes.

The main metabolic difference between the use of ^{14}C and ^{13}C-labelled subtrates is the dose of substrate used. In ^{14}C labelling studies only trace amounts of subtrate are needed due to

the high sensitivity of the beta detectors. In ^{13}C labelling studies a large amount of substrate must be used due to the relative insensitivity of the ^{13}C NMR spectrometer.

When ^{13}C-labelled precursors of the amino acids are used, a glutamine/glutamate enrichment ratio is obtained, corresponding to the RSA of glutamine obtained with radioactive precursors. As shown in Papers III, IV and V the glutamine/glutamate enrichment ratio becomes much lower than 1 with ^{13}C-labelled glucose and greatly exceeds 1 with ^{13}C-labelled acetate, showing that even when large amounts of substrate are used, compartmentation is preserved.

The large amounts of ^{13}C-labelled substrates needed in ^{13}C NMR spectroscopy may have other metabolic effects, however. Injection of large amounts of glucose may lead to a Crabtree effect (Crabtree, 1929), i.e. a decrease in the consumption of glucose and O2 along with an increase in glucose phosphorylation. A cerebral Crabtree effect was reported by Siemkovicz et al. (1982) in halothane-anesthetized rats. The lower level of glutamate and GABA in mice injected with [1-^{13}C]glucose than in mice injected with [2-^{13}C]acetate (Paper III) may be the result of a Crabtree effect. Fitzpatrick et al. (1990) did not observe any reduction in brain glutamate during infusion of [1-^{13}C]glucose (when serum glucose was ~13 mM, similar to what was obtained in Paper III) as determined by 1H NMR spectroscopy. In this context it should be noted, however, that not all pools of glutamate are accessible to 1H NMR spectroscopy (Pirttilä et al., 1993; Kauppinen et al., 1994). Transmitter glutamate is probably relatively inaccessible to 1H NMR spectroscopy (Pirttilä et al., 1993).

Injection of labelled substrates into fed animals will lead to an even higher serum glucose than when fasted animals are used. Thus 15 minutes after injection of 0.25 ml 300 mM [2-^{13}C]acetate into fasted mice, serum glucose was ~6 mM (Paper III). 15 minutes after injection into fed mice, however, serum glucose was ~13 mM (unpublished observation). When fed mice are injected with [1-^{13}C]glucose the percent enrichment of serum glucose will be lower than when the animals are fasted (cf. Van den Berg, 1973), which is why fasted animals were used in Papers III-V. In fasted animals, on the other hand, gluconeogenesis will be active, and may lead to scrambling of label from the C-1 to other positions in the glucose molecule. As noted by Fitzpatrick et al. (1990), injection of the large amount of [1-^{13}C]glucose needed for ^{13}C NMR spectroscopy, will lead to hyperglycemia and suppression of gluconeogenesis, so that redistribution of label within the glucose molecule will be minimal.

Injection of [2-^{13}C]acetate into fasted animals may lead to extracerebral conversion of acetate into glucose through gluconeogenesis. In spectra of serum extracts labelled glucose was not seen, however. Serum lactate, on the other hand, was highly labelled from [2-^{13}C]acetate in fasted animals (Paper IV). Correction for the ^{13}C-labelled lactate in the cerebral blood volume therefore had to be done. In fed animals, however, serum lactate was not labelled from [2-^{13}C]acetate, reflecting a lower activity of gluconeogenesis in the

fed state. For the sake of comparison, fasted animals were used both in experiments with ^{13}C-labelled glucose and acetate (Papers III-V). In fasted mice injected repeatedly subcutaneously with [2-^{13}C]acetate citrate labelled in the C-2 position (and/or C-4) was seen in serum extracts. C-2-labelled citrate may after cleavage yield C-3-labelled oxaloacetate and hence C-3-labelled pyruvate and lactate (Paper IV).

It has been shown that 5 mM acetate gives a 40 % inhibition of GABA transaminase purified from mouse brain (Schousboe et al., 1974). Since acetate is taken up into glia, it could be speculated that glial GABA transaminase becomes inhibited. Judging from the small peak heights of acetate C-2 in the spectra from brain extracts, however, it seems unlikely that brain acetate attained such concentrations of any duration after i.v. injection of [2-^{13}C]acetate (e.g. spectra in Papers III, IV and V). An inhibitory effect of acetate on GABA transaminase could possibly be of significance in studies on brain slices and cell cultures when such concentrations of [2-^{13}C]acetate are used (Badar-Goffer et al., 1990; 1992; Sonnewald et al., 1991; Paper II). Ex vivo exposure of the heart to acetate leads to prompt inhibition of glycolysis and a dramatic efflux of citrate (Randle et al., 1970). This may be of relevance to experiments which have shown labelling of citrate from [2-^{13}C]acetate but not from [1-^{13}C]glucose (Badar-Goffer et al., 1990), since it may be that incubation of brain tissue with [2-^{13}C]acetate induces accumulation of labelled citrate. It should be noted, however, that the citrate released from cultured astrocytes is readily labelled from [1-^{13}C]glucose (Sonnewald et al., 1991; Paper II), which shows that astrocytic accumulation and release of citrate is not merely a product of incubation with acetate. In Paper II the possible effect of acetate on the labelling from glucose and vice versa, was controlled for by coincubating cultured astrocytes with both acetate and glucose, only one of which was ^{13}C-labelled. The inhibitory effect of acetate on glycolysis (Randle et al., 1970) is not expected to affect neuronal metabolism of glucose *in vivo*, both because acetate primarily enters glia, and because the injected acetate is rapidly metabolized. Incubation of brain slices with 5 mM butyrate, which also enters glia (O'Neal et al., 1966), did not affect glucose consumption (Rolleston and Newsholme, 1967).

2.2 Nuclear magnetic resonance spectroscopy: Principles

A short and simplified description of NMR spectroscopy will be given below. Emphasis is put on aspects which are relevant to the discussion of sources of error.

^{1}H, ^{13}C and ^{31}P are among the atoms that possess nuclear spin. This can be envisaged as a circular movement of charge giving rise to an electrical current, and as such, it creates a magnetic field around itself. When subjected to an external magnetic field, the magnetic vectors of the nuclei align themselves along the external magnetic field, in a parallel or antiparallel manner, and they precess, that is, they rotate in a cone-shaped manner with a precession frequency which is dependent on the strength of the magnetic field experienced by the nuclei (Figure 2.1a). Because the parallel alignment represents a lower energy level

than the antiparallel alignment, a small majority of the nuclei prefer the parallel alignment. The vectors of the nuclei with an antiparallel alignment cancel out the vectors of the nuclei with a parallel alignment but for this little excess. Furthermore, the horizontal components of the vectors of the precessing nuclei have a random distribution, and therefore cancel each other out. The resultant longitudinal vector therefore is parallel to the external magnetic field (Figure 2.1a).

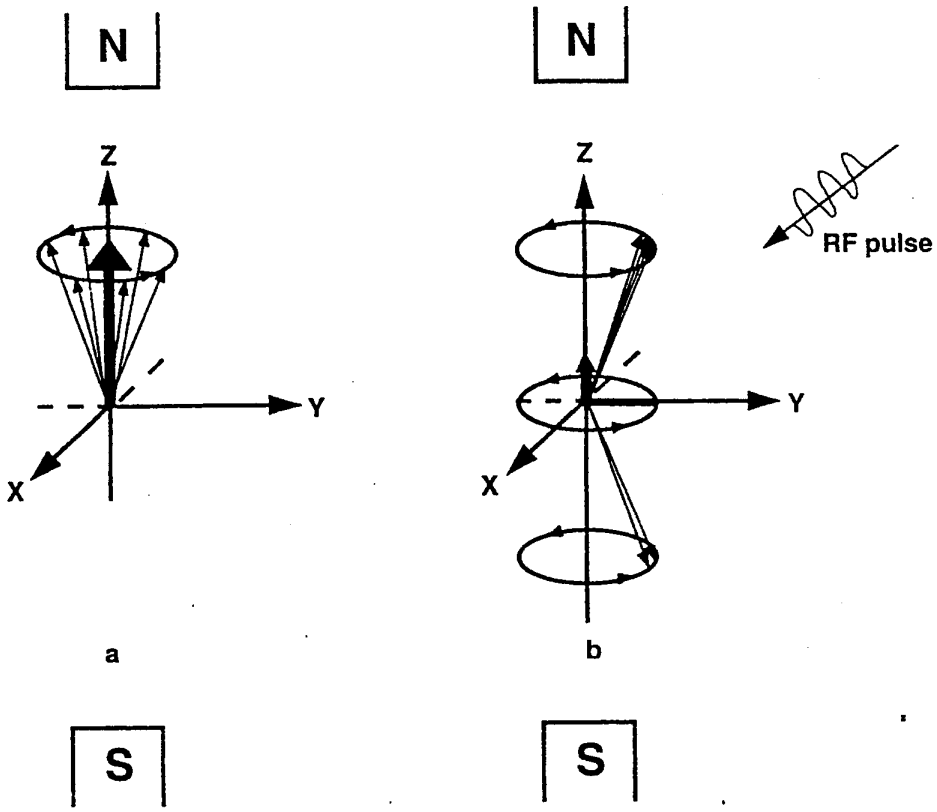

Figure 2.1a The net parallel vector (thick arrow) of the spinning nuclei in a magnetic field. The thin arrows represent the vectors of the precessing nuclei which are randomly distributed in the horizontal plane and therefore cancel each other out in that plane. N and S represent the external magnetic field.

Figure 2.1b With the application of the radiofrequency pulse (RF) the vectors of some nuclei become oriented against the magnetic field (thin arrows pointing down), which causes the parallel vector (thick arrow) to decrease. The nuclei precess in phase (thin oblique arrows), and their precession frequencies may be recorded (see Figure 2.2).

Application of a radiofrequency pulse (RF) to the magnetic field, with a frequency similar to the precession frequency, will have two effects: 1) Some of the nuclei with a parallel alignment will go from a low to a higher energy level, permitting them to shift to an antiparallel alignment, which reduces the size of the longitudinal magnetic vector. 2) The horizontal vectors of the precessing nuclei will not be randomly distributed, but will point in almost the same direction at the same time, precessing in phase, creating a rotating, horizontal magnetic vector (Figure 2.1b). As previously mentioned, the precession frequency of a nucleus depends on its surrounding magnetic field. This field consists of the external magnetic field of the NMR apparatus and of the magnetic field created by the set of electrons surrounding the nucleus, its magnetic microenvironment. The precession frequency of each nucleus differs slightly according to its magnetic microenvironment. The resulting rotating horizontal magnetic vector is therefore composed of a multitude of superimposed vectors with slightly different precession frequencies. This rotating magnetic vector may be detected by the electric current it creates in a coil, which is recorded as a decaying signal, the free induction decay (FID). The frequency, or rather frequencies, of this current equal the precession frequencies of the horizontal vectors. By Fourier transformation (FT) the FID may be converted into spectra with peaks representing individual magnetic vectors or nuclei (Figure 2.2).

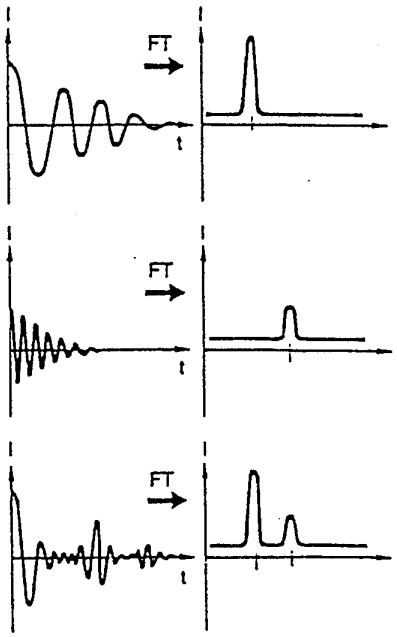

Figure 2.2 The precession frequency of a nucleus is Fourier transformed (FT) to yield a peak in an NMR spectrum (upper two panels). The lower panel shows the sum of the two upper frequencies and their transformation into two separate peaks in an NMR spectrum. The peak height is proportional to the signal intensity which (in the case of ^{13}C NMR) is proportional to the amount of ^{13}C in a specific carbon position, e.g. the amount of ^{13}C in the C-4 of glutamate or glutamine.

The integral of a peak in the spectrum is proportional to the magnitude of the vector, which again is proportional to the number of nuclei with the same magnetic microenvironment, e.g. the number of ^{13}C in the C-4 position of glutamate. To increase the signal to noise ratio the process of RF pulsing is repeated and the signals recorded via the coil, are averaged.

When the RF pulse is turned off, the nuclei relax, which means that the vectors which assumed an antiparallel alignment, return to their parallel alignment, and the horizontal vectors which precessed in phase, dephase and return to a random distribution. These processes lead to the disappearance of the horizontal vector and to the return of the parallel vector.

2.3 Sources of error in ^{13}C NMR spectroscopy

After application of the RF pulse, the electromagnetic signal is given off from a ^{13}C nucleus (and recorded via the coil) throughout the time of relaxation. It is therefore important to allow the nuclei to relax sufficiently before another RF pulse is applied. Otherwise correction factors have to be introduced to correct for the smaller signal due to the abortion of the relaxation. In the present papers the relaxation values of the nuclei investigated, i.e. the ^{13}C of CH_3, CH_2 and CH groups, was similar to that of the internal standard, dioxane, which made correction factors unnecessary. The relaxation time used in Papers II, III and IV was 3 seconds in Paper V it was 10 seconds. Experiments with longer relaxation time between the RF pulses confirmed that correction factors were not necessary. The ^{13}C of carboxyl groups, on the other hand, need much longer time to relax (cf. Badar-Goffer et al., 1990), and their use would have necessitated correction factors. Another influence on the ^{13}C signal comes from the interference of neighbouring ^{1}H, the nuclear Overhauser effect (NOE). When for instance comparing CH_3 and CH_2 groups, e.g. the C-3 and the C-2 of lactate (Papers II and IV) the three protons of the CH_3 group will give a greater contribution to the ^{13}C signal than the two protons of the CH_2 group. In Paper II factors were used to correct for this difference. In Papers III, IV and V the technique of inverse gating was used, which reduces NOE to the degree that correction is unnecessary.

With the use of internal or external standards ^{13}C NMR spectroscopy gives the total amount of ^{13}C in different carbon positions of a molecule. This permits the quantitation of ^{13}C enrichment of each carbon position without prior degradation of the molecule, as would be needed in ^{14}C labelling studies (e.g. Nicklas and Clarke, 1969). The enrichment ratios between carbon positions may be determined with one labelled precursor, whereas in ^{14}C labelling studies different animals would have to be injected with the same substrate labelled in different positions (e.g. Van den Berg et al., 1969). A critical point in ^{13}C NMR spectroscopy is to identify correctly the different peaks in the spectra. In the present papers the identification of peaks relied on previous reports (Bárány et al., 1985; London, 1988).

In some cases the assignment of peaks to specific substances was confirmed by adding the substance (succinate, malate) to the sample before obtaining spectra.

The calculation of the percent ^{13}C enrichment of various carbon positions (Badar-Goffer et al., 1990) corrects for the naturally abundant ^{13}C which is ~1.1 % of total carbon. The naturally abundant ^{13}C may contribute greatly to the peaks in the spectra, and not correcting for the naturally abundant ^{13}C may lead to erroneous interpretation of the spectra. Cerdan et al. (1990) inferred recycling of pyruvate in neurons based on the labelling pattern in glutamate obtained with [1,2-^{13}C]acetate. However, as shown by McLean et al. (1993) in a follow up experiment *in vitro*, when the natural abundance of ^{13}C was subtracted, no signs of pyruvate recycling remained.

In the ^{31}P NMR spectra of cell extracts used in Paper II, no internal standard was used, so absolute values for the different phosphorous metabolites could not be obtained. Instead the amount of ^{31}P in a peak relative to the total amount of ^{31}P in the sample was calculated. A source of error is the possible efflux of inorganic phosphate into the surrounding culture medium during fluorocitate intoxication, which would give a falsely low value both for inorganic phosphate, and for the total phosphate signal. As can be seen from Table 4 in Paper II, however, the changes in ATP and P_i with increasing concentrations of fluorocitrate are so conspicuous that efflux of P_i cannot have been of great importance.

2.4 Fluorocitrate as a gliotoxin

Fluorocitrate was used in Paper I, II and VI. Fluorocitrate is an inhibitor of aconitase (EC 4.2.1.3) of the TCA cycle (Peters, 1957). One of its isomers, (–)-erythro-fluorocitrate, is converted to the highly reactive 4-fluoroaconitate which inactivates aconitase (Villafranca and Platus, 1973; Tecle and Casida, 1989). Fluorocitrate was used in the classical studies on metabolic compartmentation in the brain because of its selective inhibitory effect on the «synthetic» TCA cycle associated with the formation of glutamine (Clarke et al., 1970; Patel and Koenig, 1971; Cheng et al., 1972). Paulsen et al. (1987) provided ultrastructural evidence for the gliotoxic, neuron-sparing action of fluorocitrate within a narrow dose range, and subsequently used fluorocitrate to study the glial-neuronal interaction in neurotransmission as revealed by microdialysis (Paulsen and Fonnum, 1989). The mechanism responsible for the glia-specific action of fluorocitrate could be the specific uptake of fluorocitrate into glia. This would be consistent with the finding that citrate is metabolized in the small compartment (Cheng, 1973; Shank and Campbell, 1984a; Paper I). Further, the precursor for fluorocitrate, fluoroacetate, which has a similar glia-specific action, acumulates in glia (Muir et al., 1986).

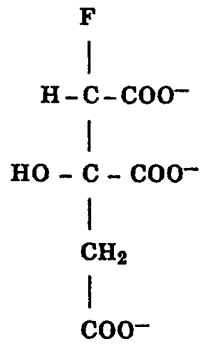

Figure 2.3 Fluorocitrate

It is not clear whether the inhibition of aconitase is responsible for all the biological effects of fluorocitrate. Goldberg et al. (1966) found that fluoroacetate, the precursor for fluorocitrate, caused a small accumulation of citrate in the brains of poisoned mice, which would be compatible with inhibition of aconitase. However, some studies have shown that (–)-erythro-fluorocitrate inhibits a citrate carrier in the mitochondrial membrane (Brand et al., 1973; Kun et al. 1977; Kirsten et al., 1978), which could also influence the cerebral level of citrate as well as the flux of metabolites through the «synthetic» TCA cycle. In addition Patel and Koenig (1971) suggested that the increase in citrate could lead to chelation of cations, which might affect enzymes which are cation-dependent, e.g. glutamine synthetase (Meister, 1980). The increase in citrate could also lead to feedback inhibition of phosphofructokinase (the Pasteur effect) with a resultant decrease in the flux through the glycolytic pathway (Goldberg et al., 1966).

In Paper VI it was shown that in astrocytes fluorocitrate caused a reduction in the formation of lactate whereas in neurons fluorocitrate caused an increase in the formation of lactate. This suggested that neurons responded to inhibition of the TCA cycle with a compensatory increase in glycolysis, whereas astrocytes did not. This may be a contributing factor in the specificity of fluorocitrate as a gliotoxin.

Recently, cytosolic aconitase was shown to be identical to an «iron-responsive element binding protein» (Kennedy et al., 1992). The iron-responsive elements themselves are stem-loop structures in untranslated regions of transferrin receptor mRNA and of the mRNA for the H-subunit of ferritin. Cytosolic, but not mitochondrial aconitase binds to these structures and regulates posttranscriptional regulation, translation and stabilization of the mRNAs in question (Haile et al., 1992; Kennedy et al., 1992; Beinert and Kennedy, 1993). In this manner cytosolic aconitase influences the intracellular levels of iron. It could be speculated that fluorocitrate might affect cytosolic aconitase, thereby disturbing intracellular iron homeostasis, which in turn could lead to cell damage through formation of free radicals.

In Paper I and VI the glia-specific action of fluorocitrate was confirmed by biochemical methods. Subsequently, the effect of fluorocitrate on the metabolism of various ^{14}C-labelled metabolites (citrate, 2-oxoglutarate, glutamate and GABA) *in vivo* was investigated to determine whether these metabolites entered a glial or a neuronal TCA cycle. Fluorocitrate was injected locally into the neostriatum to circumvent the blood-brain barrier and to avoid systemic effects. Systemic injection of the toxin in a dose that could have had cerebral effects would probably have led to cardiac arrythmias and death.

2.5 3-Nitropropionic acid

3-Nitropropionic acid (3-NPA) is an irreversible inhibitor of succinate dehydrogenase (EC 1.3.99.1) (Alston et al., 1977; Coles et al., 1979) and a reversible inhibitor of fumarase (EC 4.2.1.2) (Porter and Bright, 1980). 3-NPA is formed by several fungi and plants, and intoxication with 3-NPA is not uncommon neither in animals nor in humans (for review see Ludolph et al., 1991). In China ingestion of mildewed sugar cane has led to 3-NPA intoxication in approximately 900 people, mostly children, from 1972 - 1989. The acute symptoms include nausea, vomitting, headache, and somnolence progressing to coma. In the majority of cases the coma is reversible, but a state of chronic generalized dystonia often ensues which is due to bilateral necrosis of the basal ganglia (Ludolph et al., 1991). In rats 3-NPA leads to degeneration of the basal ganglia, an effect which is greater the older the animal (Brouillet et al., 1993). For this reason, intoxication with 3-NPA has been suggested as model for Huntington's disease which has a midlife onset (Brouillet et al., 1993; Wüllner et al., 1994). While 3-NPA intoxication may be an interesting model for Huntington's disease, it should be kept in mind that with respect to humans 3-NPA seems to be more potent as a neurotoxin in children than in adults. Further, intrastriatal injection of 3-NPA in the rat leads to depletion of dopamine (Brouillet et al., 1993), which is spared in Huntington's disease.

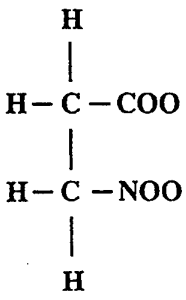

Figure 2.4 3-Nitropropionic acid

From the mechanism of action of 3-NPA, we predicted that glia would be able to compensate for the inhibition of the TCA cycle by formation of oxaloacetate via pyruvate

carboxylation, and that neurons, not expressing pyruvate carboxylase, would be selectively vulnerable.

It came as a surprise when both the glial TCA cycle and the TCA cycle associated with the large, neuronal pool of glutamate, appeared unaffected by the toxin as assessed from the metabolism of [2-^{13}C]acetate and [1-^{13}C]glucose (Paper V). From the labelling data it became clear, however, that the TCA cycle of GABAergic neurons was inhibited. This raised the question of whether GABAergic neurons have metabolic idiosyncracies which make them more vulnerable to the toxin. One such idiosyncracy could be the GABA shunt. The labelling pattern in GABA obtained during 3-NPA intoxication (Paper V) may also be consistent with inhibition of GABA transaminase or succinic semialdehyde dehydrogenase, although the build-up of succinate shows inhibition of succinate dehydrogenase. No information has been found, however, on the effect of 3-nitropropionic acid (or high levels of succinate) on GABA transaminase or succinic semialdehyde dehydrogenase of the GABA shunt. It could also be speculated that 3-nitropropionic acid is selectively taken up by GABAergic neurons and not by glutamatergic neurons or glial cells. It should be noted, however, that ^{14}C-labelled succinate, of which 3-NPA is a structure analogue, is taken up into the small compartment, and not into GABAergic neurons (Möhler et al., 1974). Propionic acid itself is also metabolized by the small compartment (O'Neal et al., 1966).

2.6 Cell cultures

In Papers II and VI cultured astrocytes and neurons were used. The *in vitro* setting allows detailed study of one or a few cell types, and it allows greater control with the metabolites entering and leaving the cells. Neural cells in culture share many of the characteristics of cells *in vivo* (Schousboe et al., 1987; Hertz et al., 1989), and extrapolation from the culture situation to the *in vivo* situation is rather common. There are obvious differences between the culture and the living brain, however. For instance, cultured neural cells are obtained from fetuses or very young animals, they grow for approximately 1 - 3 weeks in a monolayer, with a high degree of stability of the external milieu due to the composition of the culture medium. In the case of monocultures there is a lack of astrocytic-neuronal interactions, which includes trophic influences. *In vivo*, on the other hand, there is a dynamic interaction with other cell types, and the extracellular milieu changes with the neuronal activity of the tissue. Direct comparison between cultured astrocytes and neurons is also made difficult because of differences in culture conditions. For instance, the neuronal culture medium used in Paper VI, had a high concentration of potassium and a low concentration of glutamine, whereas the astrocytic culture medium was low in potassium and high in glutamine. This was necessary for the survival of the cells. However, such differences in culture conditions makes it mandatory to relate the findings *in vitro* to results obtained *in vivo*.

2.7 *Ex vivo* analysis: The problem of hypoxia and *post mortem* metabolism

In Papers I, III, IV and V labelled substrates were administered to animals, and the incorporation of label into amino acids and related metabolites was investigated after sacrifice, homogenization and extraction of brain tissue. For the results to be of relevance for the *in vivo* situation, post mortem metabolism must be prevented. In all experiments the brains were cooled or frozen in liquid nitrogen immediately after death, and homogenization was done with ice-cold trichloroacetic acid or perchloric acid. The tissue levels of amino acids hardly change within the first minute after death even without cooling (Siesjö, 1978; Perry et al., 1981). The tissue levels of glucose, lactate and TCA cycle intermediates, on the other hand, may change more rapidly (Goldberg et al., 1966; Siesjö, 1978). The level of lactate (which was measured in Papers III and IV) rises rapidly after death as a result of glycolysis (Siesjö, 1978). Reliable control values are difficult to obtain, however. The control values for brain lactate in rodents arrived at in the literature were obtained under anesthesia (Pontén et al., 1973; Siesjö, 1978), when neuronal activity and hence metabolism of glucose is depressed (See Shank et al., 1993 with respect to the metabolism of [^{13}C] glucose). Pontén et al. (1973) found that immersion of wake mice in liquid N_2 led to a level of lactate in the brain of 2.39 mmol/kg tissue wet weight, whereas N_2 immersion of mice anesthetized with N_2O/O_2, 70/30 %, gave 1.38 mmol/kg.

Attempts have been made to circumvent the problem of hypoxia, e.g. funnel freezing (Siesjö, 1978) or freeze blowing (Veech et al., 1973). Such methods involve anesthesia, however, which itself influences metabolism, as discussed above. Another problem is the small sample size which can be used; in funnel freezing only the superficial cortex can be assumed to be frozen before hypoxic conditions are established (Siesjö, 1978). This may conflict with the low sensitivity of the NMR technique for which larger amounts of tissue is needed.

3 GENERAL DISCUSSION

3.1 New aspects of the neuronal-glial interactions

The present papers add new details to the picture of glial-neuronal interactions described in the introduction. First, astrocytes continuously lose a major part of their TCA cycle intermediates, approximately 60% per turn of the cycle, mainly for the synthesis of glutamine. The loss of glial 2-oxoglutarate for the synthesis of glutamine may probably be compensated for by pyruvate carboxylation alone. Second, some of the glutamine which is transferred to GABAergic neurons, is passed through a TCA cycle before GABA is formed. From this it follows that glutamine is an energy substrate for neurons *in vivo*. It may further be speculated that the formation of transmitter amino acids from glutamine is regulated via the TCA cycle. The flux of glutamine for the synthesis of GABA alone was

assessed to be 2.2 nmol/mg protein (Paper III). This flux is very large, considering that the flux of glucose corresponds to 8 - 12 nmol/mg protein (Borgström et al., 1976; Sokoloff et al., 1977; Lu et al., 1983; Fitzpatrick et al., 1990). However, some, or perhaps most, of the glutamine which is transferred from glia to neurons is of course derived from glucose. It therefore seems that an appreciable fraction of cerebral glucose is metabolized via formation of glutamine. From the glutamine/glutamate enrichment ratio obtained with [1-^{13}C]glucose, which is 0.2 - 0.4 at 5 - 15 minutes after injection of label (Paper III), it appears that 20 - 40 % of glucose is metabolized in glia. This value agrees well with the flux of glucose through the glial TCA cycle, 27 %, which was calculated from the pyruvate carboxylase activity (Paper III). This does not mean, however, that glia extract 27 % of the energy of brain glucose. As already mentioned, glial glutamine is used by the neurons as an energy substrate.

Whereas we obtained clear evidence of transfer of glutamine from glia to neurons, trafficking of metabolites in the opposite direction was less conspicuous. The labelling of glutamine from [1-^{13}C]glucose was mainly due to glial metabolism of [1-^{13}C]glucose, not to transfer of labelled glutamate or GABA from neurons to glia. This follows from the greater labelling of glutamine C-2 than of glutamate C-2 through pyruvate carboxylase (Paper III), a precursor-product relationship similar to that seen from the labelling of glutamine and glutamate from [2-^{13}C]acetate. Further, the results of Paper I showed a predominantly neuronal uptake of exogenous GABA and glutamate, which is in accordance with previous studies (Iversen and Bloom, 1972; Gundersen et al., 1993). It may be, however, that the increase in the glutamine/glutamate enrichment ratio from 5 to 15 minutes after injection of [1-^{13}C]glucose (Paper III), indicates transfer of neuronal glutamate to glia with subsequent conversion to glutamine. It may also be that the higher C-3/C-4 labelling ratio in glutamine obtained with [1-^{13}C]glucose than with [2-^{13}C]acetate (15 minutes after injection) reflects transfer of glutamate (with a high C-3/C-4 enrichment ratio) from neurons to glia with subsequent conversion to glutamine (Paper III). On the other hand, it may be that glial uptake of neuronally derived amino acids is so small *in vivo* that it escapes detection by our methods (cf. Introduction: «Transfer of glutamate and GABA from neurons to glia»). Further *in vivo* studies, possibly involving the use of blockers of transport mechanisms, are needed to clarify this issue

From *in vitro* studies both *2-oxoglutarate*, malate (Shank et al., 1984b; Peng et al., 1991; Hertz et al., 1992) and *citrate* (Sonnewald et al., 1991) have been suggested as metabolites which are transferred from astrocytes to neurons as precursors for amino acid synthesis. The results of Paper I, in which ^{14}C-labelled 2-oxoglutarate and citrate were shown to enter glia after intrastriatal injection, do not support such a role for these metabolites. Intrastriatal injection of ^{14}C-labelled malate did also give a glutamine RSA of ~3, showing glial uptake (unpublished results). Further, cultured astrocytes did not export neither 2-oxoglutarate nor malate in NMR-detectable amounts (Paper II).

A finding which added a new dimension to the concept of glial-neuronal interactions in terms of amino acid exchange, was that of Sonnewald et al. (1993b) that cultured astrocytes convert exogenous ^{13}C-labelled glutamate largely into *lactate* and not so much into glutamine. This was also shown to be the case in brain slices (Bachelard et al., 1994). The possibility of such a pathway being operative in the intact brain was demonstrated in Paper IV, where glial formation of lactate from TCA cycle intermediates was shown. The latter pathway may be particularly important in ischemia and seizures, when extracellular levels of glutamate rise dramatically (Benveniste et al., 1984; Hagberg et al., 1985; During and Spencer, 1993). Hypoxia has been shown to cause a shift of glutamate from neurons to astrocytes in brain slices (Torp et al., 1991; 1993; Aas et al., 1993). It may be that the formation of pyruvate and hence lactate seen in Paper IV, depended on the hypoxic conditions from sacrifice until the brains became frozen (cf. Pontén et al., 1973). Conversion of TCA cycle intermediates into pyruvate has previously been thought of as a security valve to prevent the TCA cycle from being flooded with intermediates (Lehninger, 1977).

The release of *citrate* by astrocytes and not by neurons first reported by Sonnewald et al. (1991) is of interest i.a. because citrate could be an endogenous chelator of divalent cations, influencing neuronal excitability as shown by Hornfeldt and Larson (1990). Citrate could therefore be a glial neuromodulator. In Papers II and VI the large export of citrate from cultured astrocytes was confirmed. *In vivo*, however, labelling of cerebral citrate could not be detected (Papers III, IV and V). This could possibly be due to the small extracellular volume in the brain compared to the large volume of the culture medium. If citrate is released by astrocytes in the intact brain, the amounts necessary to achieve a high concentration in the extracellular fluid, could be undetectable by NMR spectroscopy.

3.2 Transmitter vs. metabolic pools of amino acids

Several attempts have been made to differentiate between the transmitter and the metabolic pools of glutamate and GABA. Engelsen et al. (1983; 1986) showed that decortication reduced the striatal level of glutamate by ~30%, and suggested that the reduction was due to the degeneration of nerve endings of glutamatergic corticostriatal fibres. Based on these and similar experiments Fonnum (1991) calculated the concentration of glutamate in glutamatergic nerve terminals to be ~40 mM. Calcium-dependency of release is commonly used to differentiate between transmitter and non-transmitter glutamate (Rubin, 1970). The presence of an amino acids in vesicles suggests its role in neurotransmission. With the use of immunocytochemistry it has been shown that potassium depolarization depletes glutamatergic nerve endings of their vesicular glutamate in a calcium-dependent manner (Storm-Mathisen and Ottersen, 1990; Laake et al., 1993). In cultured neurons depolarization with high potassium leads to preferential release of vesicular GABA, believed to be transmitter GABA, whereas depolarization with glutamate leads to relatively higher release of non-vesicular GABA (Belhage et al., 1992; 1993). Various labelling

procedures have been used to differentiate between transmitter and metabolic pools of amino acids. *In vitro* glutamine was shown to be a better precursor than glucose for releasable glutamate and GABA (Cotman and Hamberger, 1978; Tapia and Gonzalez, 1978; Hamberger et al., 1979a,b; Reubi, 1980; Ward et al., 1983). Since acetate is converted to glutamine by glia, and is transferred to neurons for the synthesis of GABA (Sonnewald et al., 1993; Paper III), it may be that the GABA and glutamate formed from the glutamine which is labelled from [^{14}C] or [^{13}C]acetate, mainly represents transmitter GABA and glutamate. The GABA and glutamate labelled from [^{14}C] or [^{13}C]glucose, on the other hand, may mainly represent non-transmitter GABA and glutamate. However, even glia might take up and metabolize glutamine (Yudkoff et al., 1988; Schousboe et al., 1993), feeding it into a glial pool of glutamate which could be different from the glutamine precursor pool (Schousboe et al., 1993). Therefore GABA remains the most reliable marker for neuronal metabolism and transmitter synthesis. In this context the observation by Muir et al. (1986) is of interest. These authors injected [^{3}H]acetate i.v. into rats, and sacrificed the animals after 30 minutes. Autoradiographic investigation of brain slices revealed no signal from nerve cell bodies, only from glia and the neuropil. At this late time point (30 min.) we know that much of the label must have been incorporated in GABA (cf. Paper III). This suggests that the GABA labelled from [^{3}H]acetate was located in nerve terminals since nerve cell bodies were not labelled from [^{3}H]acetate (Muir et al., 1986).

It is an unsettled matter whether free aspartate in the brain has metabolic roles only, or whether it may also serve as a neurotransmitter. In Paper I [^{14}C]GABA was shown to label aspartate far better than did [^{14}C]glutamate, which agrees with previous findings *in vitro* (Balázs et al., 1970). In Paper VI aspartate was shown to be especially high in cultured cortical neurons which are mainly GABAergic (Yu et al., 1984). In Paper V the labelling of aspartate from [1-^{13}C]glucose was inhibited by 3-NPA which in the same paper was shown to inhibit the TCA cycle of GABAergic neurons selectively. These findings agree with previous immunohistochemical studies showing a high level of aspartate in some populations of GABAergic neurons (Ottersen and Storm-Mathisen, 1985). The aspartate in GABAergic neurons must belong to a metabolic and not a transmitter pool. The high content of aspartate in GABAergic neurons has two interesting implications: 1) Aspartate (or aspartate labelling) may possibly be used as a marker, although not entirely specific, for GABAergic neurons. 2) During energy failure inhibitory GABAergic neurons may lose the excitatory aspartate, which may contribute to the excitotoxicity associated with ischemia. Efflux of aspartate has been shown to take place in cerebal ischemia *in vivo* (Benveniste et al., 1984; Hagberg et al., 1985).

3.3 A pool of glutamate inaccessible to ^{13}C NMR

Both *in vitro* and *in vivo* a pool of glutamate seems to escape labelling from [1-^{13}C]glucose. Incubation of cortical slices with [1-^{13}C]glucose (which yields one labelled and one unlabelled molecule of pyruvate) led to only to 15 % enrichment of glutamate C-4,

whereas lactate C-3 attained the expected ~50 % enrichment (Badar-Goffer et al., 1992). During steady state i.v. infusion of [1-^{13}C]glucose in rats an enrichment of glutamate C-4 was obtained which was only 70 % of that which would be expected from the enrichment of the serum glucose (referred by Mason et al., 1992). In the present experiments lactate C-3 and alanine C-3 were more higly enriched from [1-^{13}C]glucose than was glutamate C-4 at 5 and 15 minutes after injection (Papers III and IV), which could reflect the same phenomenon. The pool of glutamate which escapes labelling from [1-^{13}C]glucose could either be formed from unlabelled sources, or it could have a very slow turnover. The transmitter pool could, as suggested by the studies of Cotman and Hamberger (1978), be more readily labelled from glutamine than from glucose. Coincubation of brain slices with [1-^{13}C]glucose and [2-^{13}C]acetate (which labels glutamine) did, however, not increase the enrichment of glutamate in brain slices (Badar-Goffer et al., 1992). Nor does the transmitter pool have a slow turnover. Cotman and Hamberger (1978) found a very rapid synthesis in brain slices of releasable glutamate. Engelsen et al. (1983) found in decortication studies evidence for a more rapid turnover of transmitter glutamate than of metabolic glutamate *in vivo*. Recent *in vitro* studies also suggest that the turnover of glutamate in synaptic vesicles, i.e. transmitter glutamate, is very fast, with a half-life of minutes (Wang & Floor, 1994). It may be that the «glucose-inaccessible» pool of glutamate is a species-dependent phenomenon. Gruetter et al. (1994) investigated the cerebral metabolism of [1-^{13}C]glucose in healthy humans, and reported a steady state enrichment of brain glutamate C-4 which was close to the expected 50 % of the enrichment of serum glucose C-1. This indicates that in humans the glucose-inaccessible pool of glutamate is either very small or not present.

The lower than expected enrichment of glutamate in rats *in vivo* (Mason et al., 1992) raises the question of whether glucose is the sole metabolic substrate for the (rodent) brain. As mentioned earlier, the postulate that glucose is the main metabolic fuel for the brain rests mainly on the arteriovenous difference and the correlation with oxygen consumption (McIlwain and Bachelard, 1985). The present studies show, however, that acetate is metabolized by brain tissue to a high degree: The enrichment of glutamine from [2-^{13}C]acetate was similar to that of glutamate from [1-^{13}C]glucose, ~5-6 %, as calculated from the C-4 positions (Paper III). This was the case both in fed and fasted animals, and it shows that acetate was avidly metabolized by the brain. It has previously been shown that the cerebral arteriovenous difference for acetate is close to zero (Lindsay and Setchell, 1976; Pell and Bergman, 1983), which has been taken as evidence that acetate is not a metabolic fuel for the brain. It should be remembered, however, that the acetate in the venous blood may not have been the same as that in the arterial blood. It is possible for instance that some of the glucose entering the brain is converted to acetate and released to the blood stream. Conversion of

[1-^{13}C]glucose to acetate in brain slices was recently shown by Pirttilä et al. (1995), which supports the possibility that cerebral glucose may be source of acetate in the jugular vein.

3.4 The glial pool of glutamate

The pool of glutamate serving as precursor for glutamine can be estimated from the enrichment ratios in glutamate and glutamine as follows: The maximal glutamine/glutamate enrichment ratio obtained with [2-^{13}C]acetate was ~5 in the mouse (Papers III and IV), which is similar to what is found in the rat, cat and monkey (Berl et al., 1961; Berl, 1973). This would mean that the glial pool of glutamate serving as the precursor for glutamine, is 1/5 of the glial glutamine pool. In mice the total pool of glutamine is ~40 nmol/mg protein (whereas glutamate is 100 nmol/mg protein) (Papers III and V). As shown by immunocytochemistry, however, glutamine is present in neurons as well as in glia (Zhang et al., 1991; Ottersen et al., 1992). The glutamine/glutamate labelling ratio obtained with [1-^{13}C]glucose 5 minutes after i.v. injection, is 0.2, suggesting that the glial content of glutamine could be 20 % of the total glutamate, i.e.~20 nmol/mg protein (meaning that only half the glutamine in brain is in glia). This leads to a level of «glutamine precursor» glutamate in glia of ~4 nmol/mg protein (20 nmol glutamine/mg protein x 1/5). In other words, the glial pool of glutamate serving as a precursor for glutamine is ~4 % of the total pool of brain glutamate. It should be noted that this low value is valid for the mouse, which has a lower level of brain glutamine (~40 nmol/mg protein) than, e.g. the rat (~70 nmol/mg protein, Paper I). Previously a pool size of glial glutamate of ~10 % of the total brain glutamate has been calculated for the rat (Cooper et al., 1988). The low level of glial «precursor glutamate» implies that *in vivo* ^{13}C labelling studies of the large pool of glutamate (e.g. Fitzpatrick et al., 1990; Mason et al., 1992; Künnecke et al., 1993; Gruetter et al., 1994) may not have to correct for the labelling of the small pool of glutamate.

3.5 Preferential labelling of the C-2 of glutamate and glutamine from [2-^{13}C] acetate. Possible citrate lyase activity in glia and neurons *in vivo*

The higher labelling of glutamate C-2 than of the C-3 from [2-^{13}C]acetate was suggested to reflect transfer of citrate from mitochondria to cytosol in glia with subsequent cleavage to oxaloacetate and acetyl-CoA (Paper III). Van den Berg and Ronda (1976a) showed that 5 - 30 minutes after i.v. injection of [2-^{14}C]acetate into adult mice, 10 % of the radioactivity in the brain was found in lipids and 25 % in glutamate. In 10-day-old mice which have a more active lipogenesis, 30 % of the radioactivity was found in lipids (Van den Berg and Ronda, 1976b). Since acetate is metabolized in glia and not in neurons, it can be concluded that formation of fatty acids from citrate via acetyl-CoA is a rapid and prominent process in glia, and that formation of [3-^{13}C]oxaloacetate from [2-^{13}C]acetate via [2-^{13}C]citrate is equally prominent as long as [2-^{13}C]acetate is available (cf. Paper III, Fig. 5,). Metabolism of [3-^{13}C]oxaloacetate through the TCA cycle could explain the higher labelling of the C-2 than of the C-3 in glutamate (Paper III, Fig. 5). The activity of this pathway depends on the nutritional state of the animal. When fed animals were injected with [2-^{13}C]acetate, glutamine C-2 attained an enrichment which was twice as high as the C-3 at 15 minutes

(Paper IV, Table 2), whereas in fasted animals the enrichment of the two positions were not significantly different (Paper III, Table 2). This agrees well with the differential labelling of lactate C-2 and C-3 in fed and fasted animals, which was also suggested to reflect extensive metabolism of citrate through the lyase pathway in the fed state, and predominant metabolism through the TCA cycle during starvation (Paper IV).

Whether citrate cleavage takes place in neurons and to what extent, remains an open question. Transfer of citrate to the cytosol for cleavage to yield acetyl-CoA for the synthesis of acetyl choline has been suggested previously (Hayashi and Kato, 1978; Sterling and O'Neill, 1978; Sterling et al., 1981). However, [1-^{13}C]glucose injected i.v. into mice did not label GABA C-4 better than the C-3, which could have been a sign of citrate cleavage in GABAergic neurons (Paper III). Not even when mice were pretreated with 3-NPA, was there labelling of GABA C-4 from [1-^{13}C]glucose, only of the C-2 (Paper V). 3-NPA inhibits succinate dehydrogenase in GABAergic neurons, and prevents labelling of GABA C-4 via the straightforeward operation of the TCA cycle (Paper V). Taken together these observations suggests that citrate lyase is not very active in GABAergic neurons. In contrast, [1-^{13}C]glucose labelled the C-2 of glutamate significantly better than the C-3 (Paper III). This was, however, interpreted as reflecting pyruvate carboxylase activity in glia, which would also yield preferential labelling of the C-2 of glutamate. We cannot with certainty say to which degree the postulated citrate lyase activity may have contributed to the labelling of glutamate C-2 from [1-^{13}C]glucose, but it seems plausible that this labelling took place in glia and not in neurons. The reason for this is the following: The glutamine/glutamate enrichment ratio obtained with [2-^{13}C]acetate is an index of the relative labelling of glial glutamine and glutamate. If glutamate is labelled in the C-2 (through pyruvate carboxylation or citrate cleavage) from [1-^{13}C]glucose in glia, the glutamine C-2 /glutamate C-2 enrichment ratio obtained from [1-^{13}C]glucose should be the same as the ratio obtained with [2-^{13}C]acetate. On the other hand, if glutamate is labelled via citrate cleavage in neurons, the glutamine/glutamate enrichment ratio with respect to C-2 labelling should be lower than the ratio obtained with [2-^{13}C]acetate, since there would be two pools of glutamate which received preferential C-2 labelling. The glutamine/glutamate enrichment ratio obtained with [2-^{13}C]acetate was 4.7 ± 1.2 and 2.1 ± 0.2 at 5 and 15 minutes after injection, respectively (Paper III). The glutamine/glutamate enrichment ratio with respect to C-2 labelling from [1-^{13}C]glucose was 4.5 and 2.2 at 5 and 15 minutes after injection (calculated from Table 5, Paper III). These values are so similar to those obtained with [2-^{13}C]acetate, that citrate cleavage does not seem to be an important route for labelling of the large, neuronal pool of glutamate. Even in cholinergic neurons the activity of citrate lyase is low (Sterri and Fonum, 1980). Transection of cholinergic afferents to the nucleus interpeduncularis resulted in a dramatic decrease in the activity of choline acetyl transferase, a marker for cholinergic neurons, whereas citrate lyase activity was only marginally reduced (Sterri and Fonum, 1980).

In Paper VI the ^{14}C labelling of aspartate in cultured cerebellar neurons remained almost unaffected by fluorocitrate although the labelling of glutamate was significantly reduced.

This indicated a flux of ^{14}C into aspartate along pathways other than the straightforeward operation of the TCA cycle. Cultured cerebellar neurons consist mainly of glutamatergic granule cells (Schousboe et al., 1985). Both citrate lyase as well as phosphoenolpyruvate carboxy kinase could lead to labelling of aspartate from [^{14}C]glucose. The cellular distribution of these enzymes in neural cells has not been determined, and both could be active in cultured cerebellar neurons.

In Paper II, the C-2/C-4 (not the C-3/C-4) labelling ratio in glutamine was used to calculate the loss of TCA cycle intermediates per turn of the cycle. In the subsequent *in vivo* study (Paper III) it was seen that the C-2 and the C-3 of glutamate was not equally labelled from [2-^{13}C]acetate, which suggested citrate lyase activity. Possibly then, using the C-2/C-4 labelling ratio in glutamine as an index for the loss of TCA cycle intermediates from the glutamine-providing TCA cycle would lead to erroneous results since the C-2 may be labelled via pathways other than the straightforeward operation of the TCA cycle. Reanalysis of the data showed, however, that *in vitro* there was no difference between the C-2 and C-3 labelling of glutamine. Therefore the value given in Paper II for the percent loss of TCA cycle intermediates is correct. This also means that *in vitro* there was no sign of citrate lyase activity.

[1,5-^{14}C]Citrate gave very weak labelling of GABA (Paper I). This was in contrast to [U-^{14}C]2-oxoglutarate and [1-^{14}C]acetate, and was suggested to reflect differences in uptake of these metabolites by astrocytes surrounding GABAergic and glutamatergic neurons (Paper I). In view of the possible citrate lyase activity, however, an alternative explanation may be offered: When [1,5-^{14}C]citrate enters the cells it may undergo cleavage by ATP citrate lyase, yielding [1-^{14}C]acetyl-coenzyme A for fatty acid synthesis, and [4-^{14}C]oxaloacetate.

[4-^{14}C]Oxaloacetate may, after conversion to malate, be decarboxylated to pyruvate and CO_2 by the glia-specific enzyme cytosolic malic enzyme (Kurz et al., 1993), whereby the label is lost as $^{14}CO_2$. Alternatively, [4-^{14}C]oxaloacetate may enter mitochondria (as a malate or aspartate) and through metabolism through the TCA cycle yield [5-^{14}C]glutamate and

[5-^{14}C]glutamine. When this [5-^{14}C]glutamine is transferred to neurons and reconverted to [5-^{14}C]glutamate, decarboxylation to GABA will remove the ^{14}C atom. This may be why GABA is so weakly labelled by [1,5-^{14}C]citrate.

The analysis of the labelling patterns seen in the ^{13}C NMR spectra of brain extracts rely to a high degree on the notion that label is scrambled in the succinate dehydrogenase step of the TCA cycle (Papers II - V), (see Lehninger, 1977). It should be mentioned, however, that some authors entertain the idea that succinate dehydrogenase may be acting stereospecifically, i.e. that scrambling might not occur (For review, see Srere, 1990). The

equal labelling of GABA C-3 and C-4 both from ^{13}C-labelled acetate and glucose seen in Paper III suggests, however, that label was indeed scrambled.

4 PERSPECTIVES

Recently, Gruetter et al. (1994) reported on the cerebal metabolism of [1-^{13}C]glucose in healthy, unanesthetized humans as monitored by ^{13}C NMR spectroscopy. Prior to spectroscopy 1H magnetic resonance images of the brain were obtained, and a region of interest was chosen, a 6 x 4 x 6 cm3 volume in the occipital-parietal region. [1-^{13}C]Glucose was infused intravenously. Spectra were obtained with a 4-minute time resolution. Glutamate C-4 was observed in the spectra only after 4 minutes. After 1 hr glutamate C-3, glutamine C-4 and aspartate C-3 were visible. After 2 - 3 hrs glutamine C-3 was visible. Based on comparison with external standards the concentration of ^{13}C-labelled metabolites could be determined. From the glutamate C-4 isotopomers the percent enrichment of glutamate C-4 could be calculated. This study shows that detailed study of the human brain metabolism with ^{13}C NMR spectroscopy is already possible. From the labelling patterns obtained with

[1-^{13}C]glucose the same group has later calculated the rate of cerebral glucose consumption in humans, the overall cerebral TCA cycle rate, and the rate of synthesis of glutamine (Mason et al., 1995). The study by Beckman et al. (1991) may serve as an indicator of the speed of the development within this field. Three years prior to the publication of the study by Gruetter et al. (1994), Beckman et al. (1991) were not able to resolve the resonances of glutamate and glutamine labelled during infusion of [1-^{13}C]glucose, nor to study their isotopomers, in spectra obtained from the brains of healthy volunteers. The development of ^{13}C NMR spectroscopy is going to allow smaller brain volumes to be analyzed with greater metabolic detail and better time resolution. In the near future the use of different ^{13}C-labelled substrates may allow the investigation of specific metabolic pathways, some of which are specific to certain cell types. Even interactions between different cell types may studied, such as the glial-neuronal interactions investigated in the present papers. The combination of ^{13}C NMR spectroscopy with ^{31}P and 1H NMR spectroscopy will give even further metabolic detail. The relationship between metabolism and function may be investigated by stimulation of specific brain areas. (The subjects in the study by Gruetter et al. (1994) wore earplugs and eyemasks to avoid sensory stimulation.) The potentials of ^{13}C NMR spectroscopy in the investigation of brain diseases, for their early diagnosis and for the evaluation of treatment regimens seem enormous. For instance, a reduction in the cerebral metabolism of glucose as demonstrated by positron emission tomography was recently reported in asymptomatic persons at risk of developing familial Alzheimer's disease (Kennedy et al., 1995). The metabolic details of such a reduction could probably be investigated by 13C NMR spectroscopy, with greater hope of revealing the underlying metabolic deficit of that disorder.

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ERRATA

Paper I:

Introduction, 2nd paragraph, line 5: Reference 16 and 24 should be 24 and 25.

Table II, 1st column, 1st line: [U-^{14}C]Glucose.

Discussion, page 123, 1st paragraph, 2nd last line: «glutaminergic» should be «glutamatergic».

Discussion, page 123, last paragraph, line 8: «althoug» should be «although».

References, page 124, reference 23, line 4 & 5: «*Neurochem. Res.,* in press.» should be «*Neurosci. Lett.,* 128 (1991) 235-239.».

PAPER I

Brain Research, 576 (1992) 120–124

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Selective inhibition of glial cell metabolism in vivo by fluorocitrate

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The effect of fluorocitrate on glial and neuronal amino acid metabolism was studied. One nmol of fluorocitrate administered intrastriatally in the rat caused a 95% reduction of glutamine formation from [^{14}C]acetate, a substrate which enters the glial cells selectively. The metabolism of [^{14}C]glucose which enters neurons, was unaffected by fluorocitrate treatment except for the glutamine formation. This is evidence that fluorocitrate is a selective inhibitor of the glial Krebs' cycle. [^{14}C]Citrate and 2-oxoglutarate labelled amino acids in a manner similar to [^{14}C]acetate, which shows that these substrates are taken up and metabolized by glial cells. Differences in the labelling of γ-aminobutyric acid (GABA) from [^{14}C]acetate and citrate suggest that astrocytes associated with GABAergic and glutamatergic nerve terminals may differ in their preference for amino acid precursors.

INTRODUCTION

Glutamine synthesized by astroglia is thought to be an important precursor for transmitter amino acids glutamate and γ-aminobutyric acid (GABA) in vitro[9,10] as well as in vivo[17]. Glutamine itself may be synthesized from glutamate and GABA that are released from nerve endings and taken up by astrocytes, and it may be synthesized from other metabolites that feed into the glial Krebs' cycle[4,7,24].

When a radiolabelled glutamate precursor enters glial cells selectively, such as acetate[12,13], the radioactivity is incorporated into the large glial pool of glutamine, yielding a glutamine/glutamate relative specific activity (RSA) greater than one[16,24]. In contrast, a glutamate precursor such as glucose which enters neurons[12], predominantly labels the large neuronal pool of glutamate and yields a glutamine/glutamate RSA lower than one[2,6].

The study of the interaction between glial cells and neurons in terms of amino acid metabolism would be facilitated if the metabolism of one of the two cell types could be selectively inhibited. We have previously shown that fluorocitrate, a compound that blocks Krebs' cycle at the level of aconitase[20], causes ultrastructural changes in glial cells only[16], and also inhibits transmitter amino acid synthesis[17].

In this study we wished to investigate whether fluorocitrate has a selective effect on the amino acid metabolism of glial cells, and to quantify the degree of inhibition of glial glutamine formation in vivo. To do this, we measured the incorporation of radioactivity into amino acids from ^{14}C-labelled glutamate and glutamine precursors in the presence and absence of fluorocitrate. This procedure also clarified whether 2-oxoglutarate and citrate can serve as immediate precursors of the transmitter amino acids.

MATERIALS AND METHODS

Male Wistar rats weighing 200–250 g were used throughout the study. The animals were kept at 24°C with a 12 h light–dark cycle, receiving food and water ad libitum. During surgery, normal body temperature was maintained by a thermostated heating pad. Intrastriatal microinjections were done by means of a CMA/100 microinjection pump (Carnegie Medicin, Stockholm, Sweden). Labelled precursors were obtained from Dupont, NEN Products, Boston, MA, U.S.A. Specific activities were: [U-^{14}C]glucose, 258 μCi/μmol; [1-^{14}C]acetate, 55 μCi/μmol; [1,5-^{14}C]citrate, 79.3 μCi/μmol; [U-^{14}C]2-oxoglutarate, 258 μCi/μmol; [U-^{14}C]glutamate, 282 μCi/μmol; [U-^{14}C]GABA, 216 μCi/μmol. Fluorocitrate as a barium salt was obtained from Sigma Chemical Co, St. Louis, MO, U.S.A., and prepared as described by Paulsen et al.[16], to yield a 1 mM solution.

Under light ether anaesthesia holes were drilled in the skull, and 1 μl of the fluorocitrate solution or 1 μl of 0.9% NaCl was injected stereotactically into each neostriatum with the following coordinates according to Paxinos and Watson[19]: anteroposterior with respect to bregma, + 1.0 mm; lateral with respect to the midline, ± 3.0 mm; dorsoventral with respect to dura, 5.0 mm. A second microinjection was done via the same route into both neostriata after 4 h with 1 μl of either [U-^{14}C]glucose, (1.3 μCi), [1-^{14}C]acetate, [1,5-^{14}C]citrate (both 1 μCi), [U-^{14}C]2-oxoglutarate (0.6 μCi), [U-^{14}C]glutamate, or [U-^{14}C]GABA (both 0.5 μCi). The rats were decapitated 10 min after the last injection. After decapitation, the heads were cooled for 10 s in liquid nitrogen, the brains were removed rapidly and cooled for another 3 s before the neostriata were dissected out on ice and visually controlled for the injection sites.

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TABLE I

Endogenous amino acids 4 h after fluorocitrate (Fc) injection compared to controls (C)

Values are expressed as pmol/μg protein (mean ± S.E.M). n = number of neostriata.

	n	*Aspartate*	*Glutamate*	*Glutamine*	*GABA*
C	38	18.5 ± 1.0	97.8 ± 3.9	65.5 ± 2.3	18.9 ± 1.4
Fc	38	13.0 ± 0.6	83.3 ± 2.2*	34.1 ± 1.8*	16.7 ± 0.7

*$P < 0.01$.

The neostriata were homogenized in 1 ml 2.5% trichloroacetic acid which contained 40 μM α-aminoadipic acid as internal standard. The homogenates were left on ice for at least 20 min, and were then centrifuged at 20,000 *g* for 20 min. Protein content of the pellets was determined by the method of Lowry et al.[11]. The supernatant was extracted 3 times with equal volumes of ether saturated with water, passed through a Dowex 50 W column to remove radiolabelled non-amino acid metabolites, lyophilized to dryness, and redissolved in 60 μl double-distilled water. The amino acid content of the supernatants was determined by high performance liquid chromatography as described previously[16]. To determine the [^{14}C]incorporation into the various amino acids, 40 μl of the supernatant derivatized with ophthaldialdehyde reagent were passed through the same column as that used for amino acid content estimation. The supernatant was collected from the column in one minute fractions containing the separated amino acids. Their [^{14}C] content was determined by a Packard TRI-CARB 300 scintillation counter. The procedure allows the determination of the base line activity which is subtracted from the radioactivity of the eluated amino acids. Underivatized amino acids were eluated during the first five min of chromatography, before the eluation of any of the derivatized amino acids. Data were analyzed by the Student's *t*-test.

RESULTS

Four hours after microinjection of fluorocitrate, the content of endogenous glutamine was reduced to 50% of control value, while the content of aspartate, glutamate and GABA was reduced by 30, 15 and 12%, respectively (Table I).

In control neostriata, incorporation of label from [U-^{14}C]glucose gave the expected glutamine/glutamate RSA lower than one. Fluorocitrate led to a selective reduction in the specific activity of glutamine by 63%; the specific activities of aspartate and glutamate remained unchanged (Table II).

[1-^{14}C]Acetate, [1,5-^{14}C]citrate, and [U-^{14}C]2-oxoglutarate gave a glutamine/glutamate RSA greater than one (Table II).

[^{14}C]Acetate and 2-oxoglutarate gave an incorporation into GABA which was 10 times that obtained with [^{14}C]citrate (Table III). [^{14}C]citrate labelled aspartate poorly, whereas [^{14}C]2-oxoglutarate labelled aspartate well, resulting in an aspartate/glutamate RSA close to one (Table II).

In the presence of fluorocitrate the incorporation of label from [^{14}C]acetate, citrate and 2-oxoglutarate into glutamine was reduced by 95–98% (Table III). Label incorporation into aspartate, glutamate and GABA was reduced by 60–70%. The specific activities were reduced accordingly (Table II). One notable exception was the incorporation of label into aspartate from [^{14}C]2-oxoglutarate which increased 217% in the presence of fluorocitrate, resulting in an aspartate/glutamate RSA greater than 6.

^{14}C-Labelled GABA gave a glutamine/glutamate RSA of 0.77 and an aspartate/glutamate RSA greater than 2. In the presence of fluorocitrate the incorporation of label into glutamine was reduced by 75%. The radioactiv-

TABLE II

Specific activity of amino acids obtained with ^{14}C-labelled precursors 4 h after injection of fluorocitrate (Fc) compared to controls (C)

Values are expressed as dpm/pmol (mean ± S.E.M.). Numbers in parentheses are the specific activities of the amino acid relative to the specific activities of glutamate (RSA). n = number of neostriata.

		n	*Aspartate*		*Glutamate*	*Glutamine*		*GABA*	
[U-^{14}C]	C	5	0.55 ± 0.11	(0.54)	1.02 ± 0.10	0.27 ± 0.05	(0.26)		
	Fc	5	0.54 ± 0.05	(0.53)	1.01 ± 0.06	0.10 ± 0.02**	(0.10)		
[1-^{14}C]	C	6	0.10 ± 0.02	(0.31)	0.51 ± 0.10	1.22 ± 0.20	(2.39)	0.31 ± 0.05	(0.61)
Acetate	Fc	6	0.07 ± 0.00	(0.39)	0.18 ± 0.03*	0.07 ± 0.02**	(0.39)	0.10 ± 0.02*	(0.56)
[1.5-^{14}C]	C	8	0.07 ± 0.01	(0.13)	0.55 ± 0.04	1.44 ± 0.10	(2.62)	0.03 ± 0.01	(0.05)
Citrate	Fc	8	0.03 ± 0.02	(0.14)	0.21 ± 0.04**	0.13 ± 0.02**	(0.62)	0.01 ± 0.00	(0.05)
[U-^{14}C]2-Oxo-	C	7	0.41 ± 0.04	(0.95)	0.43 ± 0.04	1.26 ± 0.12	(2.93)	0.25 ± 0.03	(0.58)
glutarate	Fc	7	1.36 ± 0.15**	(6.48)	0.21 ± 0.04**	0.06 ± 0.01**	(0.62)	0.10 ± 0.03**	(0.48)
[U-^{14}C]	C	7	0.19 ± 0.07			0.98 ± 0.16		0.27 ± 0.05	
Glutamate	Fc	7	0.17 ± 0.04			0.63 ± 0.7		0.35 ± 0.07	
[U-^{14}C]	C	5	1.75 ± 0.24	(2.36)	0.74 ± 0.11	0.57 ± 0.08	(0.77)		
GABA	Fc	5	1.96 ± 0.36	(2.33)	0.84 ± 0.17	0.35 ± 0.07**	(0.42)		

*$P < 0.05$, **$P < 0.01$.

TABLE III

Incorporation of radiolabel into amino acids from ^{14}C-labelled precursors 4 h after injection of fluorocitrate (Fc) compared to control

Values are expressed as dpm/μg protein (mean ± S.E.M.). *n* = number of neostriata.

		n	*Aspartate*	*Glutamate*	*Glutamine*	*GABA*
[U-^{14}C] Glucose	C	5	8.9 ± 1.9	89.3 ± 8.1	16.4 ± 2.5	
	Fc	5	6.6 ± 0.8	77.6 ± 3.5	3.1 ± 0.3**	
[1-^{14}C] Acetate	C	6	1.9 ± 0.5	52.0 ± 12.5	85.4 ± 17.9	4.3 ± 0.8
	Fc	6	0.9 ± 0.03	15.5 ± 2.5*	4.5 ± 0.7**	1.5 ± 0.3**
[1,5-^{14}C] Citrate	C	8	1.1 ± 0.4	49.5 ± 4.8	112.7 ± 9.3	0.4 ± 0.1
	Fc	8	0.0 ± 0.0*	16.1 ± 2.6**	5.0 ± 0.7**	0.2 ± 0.0
[U-^{14}C]2-oxo-Glutarate	C	7	8.5 ± 0.8	50.3 ± 3.8	114.1 ± 7.4	4.7 ± 0.5
	Fc	7	17.6 ± 2.9*	17.7 ± 3.1**	2.2 ± 0.7**	1.8 ± 0.3**
[U-^{14}C] Glutamate	C	7	3.4 ± 1.4		63.0 ± 11.8	4.4 ± 1.1
	Fc	7	2.6 ± 0.6		16.6 ± 3.31**	6.4 ± 1.2
[U-^{14}C] GABA	C	5	34.1 ± 5.1	84.8 ± 10.8	38.1 ± 5.8	
	Fc	5	29.7 ± 6.3	75.7 ± 17.4	9.2 ± 0.7**	

*$P < 0.05$, **$P < 0.01$.

ity in GABA as a percentage of total radioactivity in the tissue increased 25% in the presence of fluorocitrate ($P < 0.01$, data not shown). This increase mainly reflects reduced metabolism of the injected [^{14}C]GABA.

[^{14}C]Glutamate strongly labelled glutamine but not aspartate. The aspartate labelling from [^{14}C]glutamate was only 10% of that obtained with [^{14}C]GABA. In the presence of fluorocitrate the incorporation of label into glutamine was reduced by 75%. The percent radioactivity in glutamate did not change significantly in the presence of fluorocitrate (data not shown).

DISCUSSION

We have shown that fluorocitrate inhibits amino acid formation from ^{14}C-labelled acetate, a substrate which is taken up and metabolized selectively by glial cells[12,13,14,24]. In contrast, amino acid formation from [^{14}C]glucose, which enters neurons[12], was uninhibited except for glutamine. This confirms the previous finding that fluorocitrate in vivo affects glial cells selectively[16].

The inhibition of glial glutamine formation by 1 nmol of fluorocitrate injected into rat neostriatum was 95–98% as judged from the incorporation of label into glutamine from [^{14}C]acetate, citrate and 2-oxoglutarate. Probably, when aconitase of the glial Krebs' cycle is blocked by fluorocitrate, the level of 2-oxoglutarate becomes so low that glutamate is fed into the Krebs' cycle at the expense of glutamine formation. Alternatively, inhibition of aconitase would ultimately deplete the glial cells of ATP, a cofactor for glutamine synthetase (EC 6.3.1.2).

The incorporation of label from [^{14}C]acetate into GABA was reduced by 75% in the presence of fluorocitrate. This is in accordance with previous findings that glutamine levels must be very low before GABA levels are severely affected[8,18].

2-Oxoglutarate and citrate were shown to enter the glial Krebs' cycle as evidenced by the glutamine RSAs of 2.9 and 2.6 obtained with [^{14}C]2-oxoglutarate and [^{14}C]citrate, respectively, and the 95–98% reduction of label incorporation into glutamine in the presence of fluorocitrate. The reduction in glutamate and GABA formation from [^{14}C]2-oxoglutarate in the presence of fluorocitrate is at variance with the proposition by Shank and Campbell[22] that 2-oxoglutarate is released from astrocytes and used by neurons as a precursor for transmitter amino acids. Our results are also at variance with the suggestion by Sonnewald et al.[23] and by Cheng[5] that citrate is taken up by neurons. If these substrates were taken up by nerve terminals as immediate transmitter precursors, fluorocitrate inhibition of glial cells would not have suppressed their conversion to glutamate and GABA.

The reduced metabolism of [^{14}C]GABA in the presence of fluorocitrate confirms that GABA is metabolized, in part, by glial cells[21]. The glutamine/glutamate RSA from [U-^{14}C]GABA was lower than one, however, in contrast with previous studies[1]. Furthermore, the amino acid labelling from [^{14}C]glutamate and GABA was similar to that obtained with [^{14}C]glucose during fluorocitrate treatment. This indicates neuronal uptake and metabolism. This probably occurred because the amounts

of [^{14}C]GABA and glutamate injected were relatively large. Also, with universally [^{14}C]-labelled GABA more intermediates of the Krebs' cycle are labelled than when for instance [1-^{14}C]GABA is used. This may have contributed to the high incorporation of label into glutamate. A large body of studies, neurochemical and morphological, has shown that, in brain, glutamate is present in various pools[7]. According to this concept, the pool of glutamate labelled by [^{14}C]acetate, citrate and 2-oxoglutarate would principally be the pool of glial glutamate through which these substrates are converted to glutamine. The glutamine can be transferred to neurons to give transmitter GABA and glutamate. The incorporation of label into GABA obtained with [^{14}C]citrate was less than 10% of that obtained with either [^{14}C]acetate or 2-oxoglutarate. This indicates a specialization of the glial cells. It may be that glial cells associated with GABAergic and glutamatergic neurons differ in their preference for glutamine precursors. Thus, citrate may preferentially enter glial cells associated with glutamatergic neurons, whereas acetate and 2-oxoglutarate may enter glial cells associated both with both glutaminergic and GABAergic neurons.

Our finding that [^{14}C]GABA labels aspartate well whereas [^{14}C]glutamate labels aspartate poorly is in accordance with the immunohistochemical demonstration that GABAergic neurons are rich in aspartate whereas glutamatergic neurons contain little aspartate[15].

[^{14}C]2-Oxoglutarate gives an aspartate/glutamate RSA close to one. The conversion of [^{14}C]2-oxoglutarate to aspartate is not inhibited, but rather enhanced by fluorocitrate treatment, which may indicate that it takes place in neurons. Possibly, 2-oxoglutarate labels a specific pool of aspartate. This could correspond to the high levels of aspartate seen in some types of neurons[15]. On the other hand, when [^{14}C]2-oxoglutarate enters the glial Krebs' cycle, it may cause a build up of oxaloacetate which would be converted to aspartate. Blocking the glial Krebs' cycle at the level of aconitase would augment the build up of oxaloacetate, leading to increased label incorporation into aspartate.

In conclusion we wish to point out that 1 nmol of fluorocitrate administered intrastriatally in the rat gives a selective inhibition of glial glutamine formation. This provides a model for the study of glia–neuron interactions, especially those pertaining to transmitter amino acid formation and recycling. We also conclude that citrate and 2-oxoglutarate are selectively taken up and metabolized by glial cells, althoug a neuronal pool of aspartate may have been directly labelled by [^{14}C]2-oxoglutarate. Finally, we propose that GABAergic and glutamatergic neurons are associated with astrocytes that differ in their preference for glutamine precursors.

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PAPER II

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NMR Spectroscopy of Cultured Astrocytes: Effects of Glutamine and the Gliotoxin Fluorocitrate

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Abstract: Glial synthesis of glutamine, citrate, and other carbon skeletons, as well as metabolic effects of the gliotoxin fluorocitrate, were studied in cultured astrocytes with ^{13}C and ^{31}P NMR spectroscopy. [2-^{13}C]Acetate and [1-^{13}C]glucose were used as labeled precursors. In some experiments glutamine (2.5 m*M*) was added to the culture medium. Fluorocitrate (20 μM) inhibited the tricarboxylic acid (TCA) cycle without affecting the level of ATP. The net export of glutamine was reduced significantly, and that of citrate increased similarly, consistent with an inhibition of aconitase. Fluorocitrate (100 μM) inhibited TCA cycle activity even more and (without addition of glutamine) caused a 40% reduction in the level of ATP. In the presence of 2.5 m*M* glutamine, 100 μM fluorocitrate did not affect ATP levels, although glutamine synthesis was nearly fully blocked. The consumption of the added glutamine increased with increasing concentrations of fluorocitrate, whereas the consumption of glucose decreased. This shows that glutamine fed into the TCA cycle, substituting for glucose as an energy substrate. These findings may explain how fluorocitrate selectively lowers the level of glutamine and inhibits glutamine formation in the brain in vivo, viz., not by depleting glial cells of ATP, but by causing a rerouting of 2-oxoglutarate from glutamine synthesis into the TCA cycle during inhibition of aconitase. Analysis of the ^{13}C labeling of the C-2 versus the C-4 positions in glutamine obtained with [2-^{13}C]acetate revealed that 57% of the TCA cycle intermediates were lost per turn of the cycle. Glutamine and citrate were the main TCA cycle intermediates to be released, but a large amount of lactate formed from TCA cycle intermediates was also released, showing that recycling of pyruvate takes place in astrocytes. **Key Words:** NMR spectroscopy—Astrocyte—Fluorocitrate—Glutamine—Citrate—Aconitase—Pyruvate carboxylase.
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In the classical studies on compartmentation of amino acid metabolism in brain, fluorocitrate was used because of its ability to inhibit a "synthetic" tricarboxylic acid (TCA) cycle selectively (Clarke et al., 1970; Patel and Koenig, 1971; Cheng et al., 1972; Clarke and Berl, 1973). The synthetic TCA cycle supplies carbon skeletons for the synthesis of glutamine, which is believed to be a main precursor for the transmitter amino acids glutamate and GABA (Hamberger et al., 1979*a,b;* Paulsen et al., 1988). With the finding that glutamine synthetase (EC 6.3.1.2) was localized in glial cells and not in neurons (Martinez-Hernandez et al., 1977; Norenberg and Martinez-Hernandez, 1979; Patel, 1982), the synthetic TCA cycle was ascribed to glial cells. It seemed likely then that fluorocitrate would predominantly affect glial cells. This was substantiated both by ultrastructural and by neurochemical studies in vivo (Paulsen et al., 1987; Hassel et al., 1992). The mechanism by which fluorocitrate inhibits glutamine synthesis is not clear. Clarke et al. (1970) showed that the activity of glutamine synthetase was unaffected by fluorocitrate. Peters (1957) originally assumed that fluorocitrate competitively blocked the enzyme aconitase (EC 4.2.1.3) of the TCA cycle. Later work showed that one of the isomers of fluorocitrate, (−)-*erythro*-fluorocitrate, is metabolized to the highly reactive 4-fluoroaconitate, which may inactivate aconitase (Villafranca and Platus, 1973; Tecle and Casida, 1989; Clarke, 1991). Kun et al. (1977), on the other hand, found that (−)-*erythro*-fluorocitrate inhibits a citrate carrier in mitochondrial membranes. This may explain why fluorocitrate inhibits bidirectional transport of citrate in mitochondria, thus affecting citrate-dependent ATP synthesis (Kirsten et al., 1978). Blocking the glial TCA cycle by fluorocitrate would be expected to alter several equilibria that are important for glutamine synthesis, as well as to affect the production of ATP, which is a cofactor for glutamine synthetase.

With NMR spectroscopy (NMRS) monitoring ^{13}C

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Abbreviations used: NMRS, NMR spectroscopy; TCA, tricarboxylic acid.

signals, changes in glycolysis and TCA cycle activity may be detected simultaneously together with changes in the levels of some associated amino acids. A unique feature of ^{13}C NMRS is the detection of ^{13}C incorporation from labeled substrates into the various positions of the molecules at study. Thus, the metabolic pathways travelled by the labeled substrates may be elucidated. With the use of NMRS of ^{31}P signals, the energy situation of the tissue may be analyzed. The aim of the present study was to investigate further the glial TCA cycle activity and to elucidate the mechanism by which fluorocitrate affects glial metabolism. We have studied the metabolism of cultured astrocytes by ^{13}C and ^{31}P NMRS in the absence and presence of fluorocitrate using [1-^{13}C]glucose and [2-^{13}C]-acetate as labeled substrates.

MATERIALS AND METHODS

Astrocytes were cultured according to the method of Hertz et al. (1989). Prefrontal cortex from newborn mice was passed through sterile Nitex nylon sieves (pore size, 80 μm) into Dulbecco's minimal essential medium containing 20% (vol/vol) fetal calf serum. The cultures were kept at 37°C in an atmosphere of 95% air/5% CO_2 and a relative humidity of 100%. The culture medium was changed twice a week. When the astrocytic cultures were 3 weeks old, they were preincubated for 2 h in Dulbecco's minimal essential medium without fetal calf serum but containing fluorocitrate in the following concentrations: 100, 20, or 0 μ*M*. The cultures were then incubated for 22 h with the same concentrations of fluorocitrate and in a similar medium without the calf serum but containing either 3 m*M* [1-^{13}C]glucose (Sigma) and 3 m*M* ^{12}C-acetate or 3 m*M* [2-^{13}C]acetate (Sigma) and 3 m*M* ^{12}C-glucose. In some experiments this medium contained 2.5 m*M* ^{12}C glutamine to elucidate the role of glutamine as a substrate for astrocytic energy metabolism.

After the incubation the culture media were removed and lyophilized. The cells were washed twice with ice-cold saline; the dishes were placed on liquid nitrogen, and 50 μl of 7% (vol/vol) perchloric acid was added. The cell–perchloric acid mixture was scraped off with a Teflon scraper, transferred to a centrifuge tube, and centrifuged at 4,000 *g* for 10 min at 4°C. The supernatant was neutralized with 9 *M* KOH and centrifuged for another 10 min. Extracts corresponding to three to 20 culture dishes were pooled and lyophilized.

Proton-decoupled 125-MHz ^{13}C NMR spectra of the culture media were obtained on a Bruker model WM-500 spectrometer operating in the Fourier transform mode with quadrature detection. Spectra were accumulated using a 30° tip angle and a spectral width of 31 KHz with 64 K data points, an acquisition time of 1.049 s, and a relaxation delay of 3 s. A line broadening of 5 Hz was used. Spectra were recorded at room temperature. The samples were prepared in D_2O, with the pH varying between 8.5 and 8.9. The number of scans was 4,000. The amount of ^{13}C-containing metabolites was assessed from their peak heights relative to that of dioxane, which was added (1% vol/vol) as an internal standard. Chemical shifts are reported relative to dioxane at 67.4 ppm. Assignments were made by comparing with extensive tabulations of chemical shifts for metabolic intermediates (Barany et al., 1985) and by recording spectra of glutamine, glutamate, glutathione, 2-oxoglutarate, citrate, and lactate under similar conditions. Forty percent of the 2-oxoglutarate is lost during lyophilization (N. Westergaard, personal communication). Some spectra were also broad band decoupled only during acquisition, which avoids nuclear Overhauser effects. From the two sets of spectra factors for the nuclear Overhauser effects of different atoms were obtained and applied to all spectra. Inversion recovery experiments were performed to obtain relaxation (T_1) values. Correction for saturation was not necessary for the resonances used for calculation because the relaxation time of dioxane was similar to that of the analyzed metabolites and because the pulse angle used was 30°. Spectra were also obtained of culture media without ^{13}C enrichment and of fresh culture medium. Only a few signals of low signal-to-noise ratio were visible between 25 and 60 ppm and did not interfere with the interpretation of the results. Chemical shift positions (in ppm) for the analyzed C atoms in glutamine and citrate are as follows: glutamine, C-2, 55.0; C-3, 27.1; C-4, 31.7; citrate, C-2 and C-4, 46.5. The ^{13}C incorporation into the C-3 position of citrate could not be quantified reliably because of a low signal-to-noise ratio due to its broad base relative to the small height. The resonance at 32.8 ppm is tentatively assigned to the glutamate C-4 in glutathione and other glutamate-containing peptides. The percent ^{13}C enrichment into the various metabolites was calculated according to the method of Badar-Goffer et al. (1990).

^{31}P NMR spectra were accumulated on the same instrument using a 30° pulse angle and a spectral width of 11 KHz with 16K data points, an acquisition time of 0.77 s, and an additional relaxation delay of 1 s. A line broadening of 5–10 Hz was used. Chemical shift positions are given relative to phosphocreatine at 0 ppm. Peak assignment was based on published chemical shift information (Glonek et al., 1982). Typically 10,000 scans were accumulated. The amount of a ^{31}P metabolite was assessed from its peak height relative to the sum of ^{31}P peak heights. Recording parameters were held constant; therefore, spectra could be compared without correcting for saturation effects.

The loss of TCA cycle intermediates per turn of the cycle was derived from the C-2:C-4 labeling ratio obtained with [2-^{13}C]acetate (see Table 3). The C-2 position in 2-oxoglutarate receives half the label in the C-4 position and half the label in the C-3 position, minus the fraction of TCA cycle intermediates that is lost per turn. Because the C-3 position also receives half the label in the C-4 position minus the fraction lost per turn, the C-2:C-4 labeling ratio can be expressed as follows:

$$\text{C-2:C-4} = \left(\text{C-4} \times \frac{F}{2} + \text{C-4} \times \frac{F^2}{4} + \text{C-4} \times \frac{F^3}{8} + \text{C-4} \times \frac{F^4}{16} \cdots\right)/\text{C-4}$$

which can be rewritten:

$$\text{C-2:C-4} = \sum_{i=1}^{n} (F/2)^i/\text{C-4}$$

where *n* is the number of turns of the TCA cycle and C-4 is the ^{13}C labeling of the C-4 position of 2-oxoglutarate and

TABLE 1. *Total amounts of various metabolites in the medium of cultured astrocytes*

Metabolite (n)	$t = 0$	Control	20 μM fluorocitrate	100 μM fluorocitrate
Glucose				
+0 Gln (7)		2,250 ± 210	3,800 ± 780	5,150 ± 730[a]
+2.5 mM Gln (4)	24,000 ± 1,600	2,230 ± 250	8,500 ± 2,100[b]	11,500 ± 2,020[a,c]
Acetate				
+0 Gln (4)	23,300 ± 970	7,980 ± 1,260	8,000 ± 1,160	11,100 ± 870
Lactate				
+0 Gln (7)		29,500 ± 3,900	19,000 ± 1,640	22,100 ± 1,650
+2.5 mM Gln (4)		28,700 ± 6,900	22,300 ± 3,620[b]	13,200 ± 700[a,c]
Glutamine				
+0 Gln (8)		1,730 ± 140	1,140 ± 100[a]	485 ± 20[a]
+2.5 mM Gln (4)	20,800 ± 970	11,500 ± 1,450	6,840 ± 2,050	5,700 ± 620[b]
GSH				
+0 Gln (8)		360 ± 35	240 ± 30[b]	170 ± 10[a]
+2.5 mM Gln (4)		420 ± 20	410 ± 60[d]	315 ± 30[b,c]
Alanine				
+0 Gln (8)		450 ± 50	460 ± 35	370 ± 20
+2.5 mM Gln (4)		980 ± 90[c]	1,140 ± 200[c]	810 ± 70[c]
Citrate				
+0 Gln (8)		870 ± 120	1,690 ± 130[a]	2,000 ± 100[a]
+2.5 mM Gln (4)		800 ± 170	2,230 ± 250[a]	3,100 ± 270[a,c]

Total amounts of metabolites in the culture medium of astrocytes incubated for 22 h with 0, 20, or 100 μM fluorocitrate (plus a 2-h preincubation period) in the absence or presence of 2.5 mM glutamine (Gln). All cultures were also incubated with 3 mM glucose and 3 mM acetate, one of which was ^{13}C-labeled. The column with the heading $t = 0$ shows values at the start of the incubation. Data are mean ± SEM values, in nmol/mg of protein.

[a] $p < 0.01$, [b] $p < 0.05$ for difference from control.

[c] $p < 0.01$, [d] $p < 0.05$ for difference from cultures that were not supplemented with glutamine.

glutamine. With a continuous supply of ^{13}C-labeled substrates, the C-4 is a constant value; F is the fraction of the TCA cycle intermediates that remains in the cycle per turn. The division by 2 comes from the scrambling of label in the symmetrical succinate step (cf. Fig. 1).

The total amounts of amino acids were analyzed by HPLC after derivatization with *o*-phthaldialdehyde using α-aminoadipic acid as the internal standard. Glucose and lactate were quantified by reflectance spectrophotometry at 555 nm using a Kodak Ektachem DT 60 II analyzer. Citrate was quantified spectrophotometrically (kit no. 139 076; Boehringer-Mannheim). This method did not detect levels of fluorocitrate as high as 1 mM, which is 10 times higher than the highest concentration used in these experiments. Acetate was quantified from the ^{13}C NMR spectra. Protein content was analyzed according to the method of Lowry et al. (1951).

RESULTS

Total amounts of metabolites in culture medium: Effects of fluorocitrate on astrocyte metabolism in the absence and presence of glutamine

The astrocytic cultures were supplemented with glucose and acetate, each at 3 mM, which corresponds to 24,000 nmol/mg of protein. As can be seen from Table 1, 90% of the glucose and 60% of the acetate were metabolized after 22 h under control conditions. Fluorocitrate reduced the metabolism of glucose dose dependently, whereas the metabolism of acetate was not significantly altered. Fluorocitrate caused a dose-dependent reduction in the export of glutamine, whereas that of citrate was correspondingly increased, suggesting a block of the TCA cycle beyond the synthesis of citrate but before formation of 2-oxoglutarate. Some cultures were supplemented with 2.5 mM glutamine, which corresponds to 20,000 nmol/mg of protein. After 22 h under control conditions half the glutamine remained. Addition of glutamine did not affect the metabolism of glucose, lactate, or citrate in the control situation, whereas the level of alanine was higher with than without glutamine supplementation. In the presence of fluorocitrate the consumption of the added glutamine increased, whereas the metabolism of glucose decreased. This shows that glutamine substituted for glucose as an energy substrate. In agreement with this, less lactate and more citrate were produced when glutamine was added in the presence of fluorocitrate. Glutamine supplementation ameliorated the decrease in the level of glutathione caused by fluorocitrate.

Metabolites in astrocytic culture medium labeled from [2-^{13}C]acetate: Effects of fluorocitrate

[2-^{13}C]Acetate labeled glutamine, citrate, lactate (Table 2), and glutamate-containing peptides. The ^{13}C enrichment of the C-4 position of glutamine reached 2.36%, as opposed to 0.67% for the C-2 position. This is in agreement with the entry of [2-^{13}C]-acetate into the TCA cycle as acetyl-CoA, by which it labels the C-4 position in 2-oxoglutarate and gluta-

TABLE 2. *Percent enrichment from [2-^{13}C]acetate and [1-^{13}C]glucose into various metabolites in the culture medium of astrocytes in the absence and presence of fluorocitrate and glutamine*

Metabolite (n)	0 μM fluorocitrate		20 μM fluorocitrate		100 μM fluorocitrate	
	C-2	C-4	C-2	C-4	C-2	C-4
Glutamine						
[2-^{13}C]Acetate (5)	0.67 ± 0.16	2.36 ± 0.34	0.59 ± 0.34	1.03 ± 0.43	ND	ND
[1-^{13}C]Glucose (6)	3.36 ± 0.19[a,b]	2.98 ± 0.37[b]	1.91 ± 0.80	1.67 ± 0.86	ND	ND
[1-^{13}C]Glucose + 2.5 mM Gln (4)	0.53 ± 0.35	0.91 ± 0.48	0.29 ± 0.13	0.77 ± 0.26	ND	0.45 ± 0.22
Citrate	C-2 + C-4		C-2 + C-4		C-2 + C-4	
[2-^{13}C]Acetate (4)	12.0 ± 2.6		5.6 ± 0.25		3.5 ± 0.34[c]	
[1-^{13}C]Glucose (4)	11.9 ± 1.6		12.1 ± 1.6		10.0 ± 0.6[a]	
[1-^{13}C]Glucose + 2.5 mM Gln (4)	11.3 ± 2.5		6.6 ± 1.3		8.2 ± 0.6[a]	
Lactate	C-2	C-3	C-2	C-3	C-2	C-3
[2-^{13}C]Acetate (4)	0.23 ± 0.08	0.12 ± 0.06	0.39 ± 0.14	ND	0.10 ± 0.06	ND
[1-^{13}C]Glucose (4)	1.27 ± 0.26	30.6 ± 2.0	1.29 ± 0.40	26.4 ± 1.4	0.47 ± 0.27	22.4 ± 4.9
[1-^{13}C]Glucose + 2.5 mM Gln (4)	0.93 ± 0.38	27.0 ± 2.5	1.12 ± 0.19	23.3 ± 1.7	1.11 ± 0.42	20.6 ± 1.9
Alanine	C-3		C-3		C-3	
[2-^{13}C]Acetate (4)	ND		ND		ND	
[1-^{13}C]Glucose (4)	13.3 ± 0.9[d]		ND		ND	
[1-^{13}C]Glucose + 2.5 mM Gln (4)	20.1 ± 2.4		15.4 ± 2.1		12.1 ± 1.0[c]	

Data are mean ± SEM values. Gln, glutamine; ND, not detected.
[a] $p < 0.01$ for difference from value obtained with [2-^{13}C]acetate.
[b] $p < 0.01$, [d] $p < 0.05$ for difference from Gln-supplemented cultures.
[c] $p < 0.05$ for difference from control.

mine (Fig. 1). The C-2 position in glutamine is labeled by passage of the carbon skeleton through the TCA cycle. The C-2:C-4 labeling ratio for glutamine was 0.27 (Table 3). Using the formula for the C-2:C-4 labeling ratio as a function of the fraction of the TCA cycle intermediates that remains in the cycle each turn (see Materials and Methods), it was calculated that 57% of the TCA cycle intermediates are lost from the TCA cycle each turn. In the presence of fluorocitrate the ^{13}C labeling of glutamine was reduced dose dependently; with 100 μM fluorocitrate no ^{13}C-labeling of glutamine could be detected. However, in the presence of 20 μM fluorocitrate the C-2:C-4 labeling ratio for glutamine was similar to the control, suggesting that the fraction of the TCA cycle intermediates lost per turn of the cycle was the same as under control conditions.

Because citrate is symmetrical, the signals from the C-2 and the C-4 positions fall together into one peak. The sum of the percent enrichment of both peaks was 12%. This would maximally correspond to a net export of 104 nmol of [^{13}C]citrate over 22 h (calculated as percent enrichment × total amount; see Tables 1 and 2) if all the label were in the C-2 position only. The percent enrichment was reduced dose dependently in the presence of fluorocitrate (Table 2).

Lactate was weakly labeled in the C-2 position, a sign that TCA cycle intermediates left the TCA cycle to form pyruvate and hence lactate, a phenomenon referred to as recycling of pyruvate (Cerdan et al., 1990). The glutamate-containing peptides could not be analyzed in detail. Preliminary investigation with mass spectroscopy showed that glutathione could account for some but not all the signal in this peak.

Metabolites in astrocytic culture medium labeled from [1-^{13}C]glucose: Effects of fluorocitrate and glutamine

The ^{13}C enrichment of the C-4 position of glutamine obtained with [^{13}C]glucose reached 3%, which corresponds to a net export of 50 nmol/mg of protein of [4-^{13}C]glutamine to the culture medium over 22 h. This was similar to the incorporation obtained with [^{13}C]acetate (Table 2), which shows that the astrocytes did not prefer one substrate to the other for the formation of acetyl groups. However, [^{13}C]glucose labeled the C-2 position of glutamine five times more strongly than did [^{13}C]acetate, the C-2:C-4 labeling ratio reaching 1.18 (Table 3). This is a sign that [^{13}C]glucose to some extent entered into the TCA cycle by a pathway different from that used by [^{13}C]acetate, which in brain tissue is the carboxylation of pyruvate to oxa-

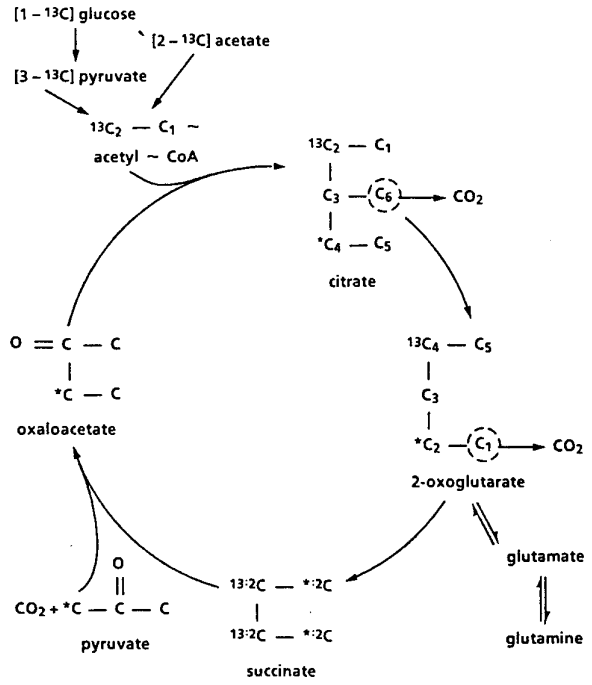

FIG. 1. A simplified scheme of the TCA cycle shows the conversion of [1-^{13}C]glucose and [2-^{13}C]acetate to [2-^{13}C]acetyl-CoA and hence the labeling of the C-2 position in citrate and the C-4 position in 2-oxoglutarate. Because succinate is symmetrical, the label is distributed equally between the C-2 and the C-3 positions (symbolically 13:2) before formation of oxaloacetate. [1-^{13}C]-Glucose yields [3-^{13}C]pyruvate, which also may form oxaloacetate after carboxylation. This produces C-2-labeled oxaloacetate (asterisk represents ^{13}C). In succinate this label is distributed equally (*:2) between the C-1 and C-4 positions.

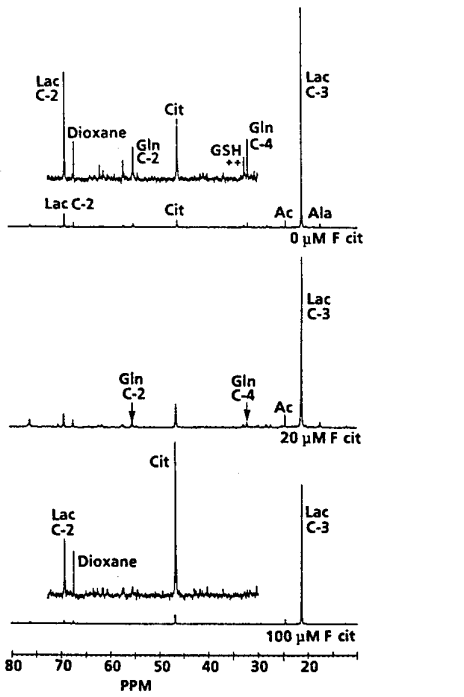

FIG. 2. ^{13}C spectra of the media of cultured astrocytes incubated for 22 h with 3 m*M* [1-^{13}C]glucose and 3 m*M* [^{12}C]acetate, without glutamine supplementation but with 0, 20, or 100 μM fluorocitrate (F cit). **Insets:** 10× magnifications of the corresponding spectra. Lac C-2 and Lac C-3, the C-2 and the C-3 of lactate; Gln C-2 and Gln C-4, the C-2 and the C-4 of glutamine; Cit, the sum of the signals from the C-2 and the C-4 positions in citrate; GSH++, glutathione and other glutamate-containing peptides; Ac, the C-2 of acetate; Ala, the C-3 of alanine.

loacetate by pyruvate carboxylase (EC 6.4.1.1) (Salganicoff and Koeppe, 1968; Patel, 1974). Carboxylation of [3-^{13}C]pyruvate leads to labeling of the C-2 position in 2-oxoglutarate and hence in glutamine (see Fig. 1). With addition of glutamine (2.5 m*M*) the C-2:C-4 labeling ratio in glutamine was reduced to 0.39 (Table 3). This shows that addition of glutamine inhibited the pyruvate carboxylation associated with formation of glutamine. On the other hand, the ^{13}C incorporation into the C-4 of glutamine was 0.91% when glutamine was added, which corresponds to a net export of 105 nmol/mg of protein of [4-^{13}C]-glutamine. This is twice the amount exported without glutamine supplementation and probably reflects the dilution of the exported [^{13}C]glutamine, which reduced its reuptake and metabolism. Fluorocitrate reduced the ^{13}C incorporation into glutamine dose dependently (Table 2 and Fig. 2). With 100 μM fluorocitrate incorporation was only detectable in the C-4 positions when the cultures were supplemented with glutamine.

TABLE 3. *C-2:C-4 labeling ratios for glutamine (Gln)*

Precursor (no. of experiments)	Fluorocitrate 0 μM	Fluorocitrate 20 μM
[2-^{13}C]Acetate (5)	0.27 ± 0.028[a]	0.26 ± 0.17
[1-^{13}C]Glucose (6)	1.18 ± 0.11	0.60 ± 0.29
[1-^{13}C]Glucose + 2.5 m*M* Gln (4)	0.39 ± 0.019[a]	0.39 ± 0.036

The C-2:C-4 labeling ratio is obtained by dividing the percent enrichment of the C-2 position in Gln by that of the C-4 position for each NMR spectrum. In the presence of 100 μM fluorocitrate incorporation of ^{13}C was only detectable in the C-4 position of Gln in cultures that were supplemented with 2.5 m*M* Gln.

[a] $p < 0.01$ for difference from value obtained with [1-^{13}C]glucose without Gln supplementation.

[^{13}C]Glucose labeled citrate (measured as the sum of the labeling of the C-2 and the C-4 positions) to the same degree as did [^{13}C]acetate, but in contrast to the latter, the percent enrichment obtained with [^{13}C]-glucose did not decrease with addition of fluorocitrate (Table 2). This indicates that, in the presence of fluorocitrate, [^{13}C]glucose entered into the TCA cycle by a

TABLE 4. *Phosphorous metabolites in perchloric acid extracts of astrocytic cultures*

Fluorocitrate (no. of experiments)	ATP γ	PCr	P_i	GPC	PC
0 μM					
+0 Gln (4)	9.3 ± 1.0	6.6 ± 0.076	20.3 ± 2.5	9.3 ± 1.3	6.5 ± 0.1
20 μM					
+0 Gln (4)	8.3 ± 1.7	5.87 ± 0.35	20.9 ± 1.9	12.4 ± 2.5	7.3 ± 0.2[a]
+2.5 mM Gln (1)	10.9		16.9		6.7
100 μM					
+0 Gln (4)	5.6 ± 1.1[a]	6.1 ± 0.58	37.5 ± 2.2[b]	8.1 ± 0.9	9.8 ± 0.5[b]
+2.5 mM Gln (1)	10.0		14.7		7.2

Data are mean ± SEM values, given as the signal intensities of the peaks as a percentage of the total phosphorous signal in the spectrum. ATPγ, γ-phosphate group of ATP; PCr, phosphocreatine; P_i inorganic phosphate; GPC, glycerol 3-phosphorylcholine; PC, phosphorylcholine; Gln, glutamine.

[a] $p < 0.05$, [b] $p < 0.01$, difference from control by t test.

pathway not used by [^{13}C]acetate, viz., by carboxylation of [^{13}C]pyruvate to oxaloacetate. Addition of glutamine did not affect the incorporation of ^{13}C from [^{13}C]glucose into citrate.

Lactate was heavily labeled in the C-3 position, as expected from glycolytic metabolism of glucose. [^{13}C]-Glucose also labeled the C-2 position of lactate with a ^{13}C enrichment of 1.27%. This corresponds to a net export of 375 nmol/mg of protein of [2-^{13}C]lactate to the culture medium over 22 h. This lactate must have been formed from TCA cycle intermediates and shows recycling of pyruvate (Cerdan et al., 1990). As for glutamine, labeling of the C-2 of lactate was five times higher with [^{13}C]glucose than with [^{13}C]acetate. This shows that the lactate and glutamine were exported from a TCA cycle whose pool of oxaloacetate is highly dependent on pyruvate carboxylation.

[1-^{13}C]glucose also labeled alanine in the C-3 position (Table 2). With glutamine supplementation the incorporation increased, suggesting that increased metabolism of glutamine through 2-oxoglutarate enhances transamination of pyruvate to alanine. In the presence of 100 μM fluorocitrate labeling of alanine was only seen when glutamine had been added to the culture medium.

^{31}P NMRS of cell extracts

Fluorocitrate at 20 μM did not affect the levels of phosphocreatine, ATP, or inorganic phosphate (Table 4). Without glutamine supplementation 100 μM fluorocitrate caused a 40% reduction in ATP level and a 68% increase in inorganic phosphate level. It is interesting that the level of phosphocreatine remained unaltered. With glutamine supplementation no changes were seen in the levels of these metabolites even in the presence of 100 μM fluorocitrate. The level of glycerol 3-phosphorylcholine, a marker for lipid metabolism (Merchant et al., 1988), was not changed significantly by fluorocitrate, whereas the level of phosphorylcholine increased dose dependently with addition of fluorocitrate.

DISCUSSION

The mechanism behind the reduced formation of glutamine during treatment with fluorocitrate was a main interest in the present study. With 20 μM fluorocitrate the formation of glutamine was markedly inhibited, although ATP was maintained at control levels. The ATP requirement of glutamine synthetase was thus probably unaffected under these conditions. There was, however, a buildup of citrate, indicating a block of the TCA cycle and a reduced synthesis of 2-oxoglutarate, which is likely to have affected glutamine formation. In fact, the increased metabolism of the added glutamine in the presence of fluorocitrate shows that rather than being formed, glutamine was consumed to supply the TCA cycle with carbon skeletons. The further reduction in glutamine synthesis seen with 100 μM fluorocitrate could in part be caused by the accompanying decrease in ATP levels, which may have reduced the activity of glutamine synthetase. In addition, the increased level of phosphate in the presence of 100 μM fluorocitrate may have augmented the activity of phosphate-activated glutaminase (EC 3.5.1.2), also stimulating consumption of glutamine. Maintaining ATP and phosphate levels by addition of 2.5 mM glutamine restored glutamine synthesis to some extent.

The increase in citrate content caused by fluorocitrate was dependent on pyruvate carboxylase activity, as indicated by the higher incorporation into citrate from [^{13}C]glucose than from [^{13}C]acetate. The actual reduction in incorporation into citrate from [^{13}C]-acetate in the presence of fluorocitrate suggests increased production of unlabeled acetyl-CoA.

A controversy exists in the literature as to whether fluorocitrate primarily blocks aconitase or a mitochondrial transport system for citrate (Brand et al., 1973; Kun et al., 1977; Kirsten et al., 1978; Clarke, 1991). The measured increase in the level of citrate in the culture medium shows that under the present conditions the transport of citrate out of mitochondria

was probably not inhibited by fluorocitrate, as maintained by Kun et al. (1977) for a mitochondrial preparation.

It is interesting that the level of ATP decreased in the presence of 100 μM fluorocitrate whereas the level of phosphocreatine remained constant. This is in contrast to what is seen under hypoxic or hypoglycemic conditions, in which the phosphocreatine level falls (Cox et al., 1988), and may indicate an inhibition of creatine kinase, which transfers phosphate from phosphocreatine to ADP. This reaction is pH dependent, and it may be that pH in our experiments increased with fluorocitrate because the production of lactate was reduced.

A ^{13}C enrichment of 3% in the C-4 position of glutamine (Table 2) is surprisingly low considering the high concentration of labeled precursors, and it indicates the presence of other sources of unlabeled acetyl-CoA or 2-oxoglutarate. An unlabeled source also provides pyruvate, which is metabolized to lactate, as the ^{13}C enrichment into the C-3 of lactate is ~30% in the control situation, as opposed to the expected 50% if [1-^{13}C]glucose were the only source of pyruvate. Because the label that enters the TCA cycle is diluted to a much greater extent than the label that forms lactate, it follows that the source of the dilution supplies carbon skeletons that preferentially enter the TCA cycle. The culture medium contains 14 different amino acids. Most of them are metabolized through the TCA cycle after conversion to acetyl-CoA, e.g., leucine and lysine, or to 2-oxoglutarate, e.g., arginine and histidine, without prior formation of pyruvate. Some of the amino acids (glycine, threonine, and serine) form pyruvate, however. Metabolism of unlabeled amino acids may explain the dilution of the label in glutamine and lactate.

The C-2:C-4 labeling ratio in glutamine obtained with [2-^{13}C]acetate after 22 h of incubation is used to calculate the percent loss of TCA cycle constituents per turn of the cycle. Our value is the same as that obtained after 17 h of incubation (U. Sonnewald et al., unpublished data). There was no shortage of [^{13}C]-acetate in our experiment because 40% of the acetate remained in the culture medium after 22 h. It is therefore likely that we have a steady-state situation in our experiments with regard to the metabolism of acetate.

The labeling of the C-2 versus the C-4 position in glutamine obtained with [1-^{13}C]glucose compares well to results from a 48-h incubation under similar conditions (Sonnewald et al., 1991). The additional labeling of the C-2 position from [^{13}C]glucose compared with that obtained with [^{13}C]acetate therefore reflects the activity of pyruvate carboxylase and not just a shortage of [^{13}C]glucose, which would have given glutamine predominantly labeled in the C-2 position (van den Berg, 1973).

The demonstration of a TCA cycle that loses more than half of its intermediates per turn, partly for the synthesis of glutamate and glutamine, fits well with the classical description of a "synthetic" TCA cycle (Berl, 1973). The association of this cycle with pyruvate carboxylation agrees with the notion of such carboxylation as an important prerequisite for glutamine formation (Waelsch et al., 1964). Also, in the guinea pig renal cortex, formation of glutamine from pyruvate has been shown to depend on pyruvate carboxylase (Forissier and Baverel, 1981; Michoudet et al., 1988). In human deficiency of pyruvate carboxylase the levels of glutamine are low in brain and CSF (Perry et al., 1985). The observed inhibitory effect of glutamine supplementation on pyruvate carboxylation suggests that the carboxylation is tuned to the demand for glutamine synthesis. This is also suggested by studies on brain slices in which K^+ depolarization, which increases the release of glutamine and glutamine-derived glutamate (Cotman and Hamberger, 1978), stimulates pyruvate carboxylase activity (Kaufman and Driscoll, 1992) and augments glial glucose consumption (Badar-Goffer et al., 1992). In this context it is noteworthy that glutamate, which is in equilibrium with glutamine, has been shown to inhibit pyruvate carboxylase in the mammalian liver in physiological concentrations (Scrutton and White, 1974).

Glutamine and citrate appear to be the main constituents of the TCA cycle that are exported from astrocytes in culture. Citrate is labeled equally well from [^{13}C]glucose and [^{13}C]acetate, whereas [^{13}C]glucose labels glutamine twice as well as does [^{13}C]acetate. This is surprising because glutamine is formed from citrate, and it may suggest that the citrate and glutamine that are exported are synthesized in different compartments. This is currently under study in our group.

Lactate formed from [1-^{13}C]glucose through glycolysis would only be labeled in the C-3 position. The finding that astrocytes export lactate labeled in the C-2 position from [1-^{13}C]glucose shows that TCA cycle intermediates that may form pyruvate (malate, oxaloacetate, or citrate) are lost from the cycle and converted to lactate before being exported out of the cells. If the 375 nmol/mg of protein of [2-^{13}C]lactate corresponds to a percent enrichment similar to that of glutamine or citrate (3–12%), then the total export of lactate derived from TCA cycle intermediates is quite large (3–11 μmol/mg of protein), and it will make a substantial contribution to the total level of lactate (29 μmol/mg of protein) in the culture medium. Cerdan et al. (1990) described a "pyruvate recycling" in neurons in vivo. The present study shows that a similar phenomenon is found in astrocytes as well.

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PAPER III

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Glial–Neuronal Interactions as Studied by Cerebral Metabolism of [2-^{13}C]Acetate and [1-^{13}C]Glucose: An Ex Vivo ^{13}C NMR Spectroscopic Study

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Abstract: Mice were injected intravenously with [2-^{13}C]-acetate or [1-^{13}C]glucose and killed after 5, 15, or 30 min. Another group of animals was injected three times subcutaneously during 30 min with [2-^{13}C]acetate to achieve a steady-state-like situation. Brain extracts were analyzed by ^{13}C NMR spectroscopy, and the percent enrichment of various carbon positions was calculated for amino acids, lactate, and glucose. Results obtained with [2-^{13}C]acetate, which is metabolized by glia and not by neurons, showed that glutamine originated from a glial tricarboxylic acid cycle (TCA cycle) that loses 65% of its intermediates per turn of the cycle. This TCA cycle was associated with pyruvate carboxylation, which may replenish virtually all of this loss, as seen from the labeling of glutamine from [1-^{13}C]glucose. From the C-3/C-4 labeling ratios in glutamine and glutamate and from the corresponding C-3/C-2 labeling ratio in GABA obtained with [2-^{13}C]acetate, it was concluded that the carbon skeleton of glutamine to some extent was passed through TCA cycles before glutamate and GABA were formed. Thus, astrocytically derived glutamine is not only a precursor for transmitter amino acids but is also an energy substrate for neurons in vivo. Furthermore, the neuronal TCA cycles may be control points in the synthesis of transmitter amino acids. Injection of [2-^{13}C]acetate led to a higher ^{13}C enrichment of the C-2 in glutamate and of the corresponding C-4 in GABA than in the C-3 of either compound. This could reflect cleavage of [2-^{13}C]-citrate and formation of [3-^{13}C]oxaloacetate and acetyl-CoA, i.e., the first step in fatty acid synthesis. [3-^{13}C]-Oxaloacetate would, after entry into a TCA cycle, give the observed labeling of glutamate and GABA. **Key Words:** ^{13}C NMR spectroscopy—Cerebral metabolism—Astrocytes—Neurons—Glutamate—GABA.
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^{13}C nuclear magnetic resonance (NMR) spectroscopy allows the detection of ^{13}C incorporation from ^{13}C-labeled precursors into the various carbon positions of small metabolites, such as tricarboxylic acid cycle (TCA cycle) intermediates and amino acids. Which carbon positions are to be labeled depends on the metabolic pathways traveled by the ^{13}C atoms. Therefore, from the labeling pattern in the metabolites, inferences can be made about the metabolic pathways in operation.

Several recent in vivo and ex vivo studies with ^{13}C NMR spectroscopy have confirmed previous concepts of cerebral metabolism, such as the existence of more than one cerebral pool of glutamate (Cerdan et al., 1990; Künnecke et al., 1993) and the association of glutamine formation with pyruvate carboxylation (Künnecke et al., 1993; Shank et al., 1993), which was first identified in ^{14}C incorporation studies (for reviews, see Berl, 1973; Van den Berg, 1973). Furthermore, studies using brain slices incubated with [1-^{13}C]glucose have demonstrated a pool of glutamate that escapes ^{13}C NMR detection (Badar-Goffer et al., 1990, 1992; Kauppinen et al., 1994). This pool could correspond to the vesicular pool of glutamate (Badar-Goffer et al., 1992; Pirttilä et al., 1993). ^{13}C NMR spectroscopy has also unveiled new aspects of cerebral metabolism, such as the release of citrate from cultured astrocytes (Sonnewald et al., 1991; Hassel et al., 1994) and the formation of pyruvate from TCA cycle intermediates as seen in vivo (Cerdan et al., 1990; Künnecke et al., 1993) as well as in vitro (Sonnewald et al., 1993*a*; Hassel et al., 1994).

Acetate is generally considered to be metabolized by glia and not by neurons, whereas glucose is metabolized by both cell types but chiefly by neurons (Minchin and Beart, 1975; Muir et al., 1986; Sonnewald et al., 1991; Badar-Goffer et al., 1992; Hassel et al., 1992). This assumption forms the basis for the following work. The cerebral metabolism of [2-^{13}C]acetate and [1-^{13}C]glucose was investigated in the unanesthe-

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Abbreviations used: NMR, nuclear magnetic resonance; TCA cycle, tricarboxylic acid cycle.

tized mouse to identify differences in glial and neuronal metabolism as well as to trace the interchange of carbon skeletons between these metabolic compartments.

MATERIALS AND METHODS

Materials

[2-^{13}C]Acetate and [1-^{13}C]glucose (99% ^{13}C enrichment) were obtained from Sigma (St. Louis, MO, U.S.A.). NMRI mice (Bomholt, Ry, Denmark) were kept under conditions of constant temperature (21°C) and humidity (50%), with a 12-h light/dark cycle and free access to food and water.

Experimental animals

Mice (20–25 g body weight) were fasted overnight, restrained, and injected intravenously over 5 s in a tail vein with 0.25 ml of 300 m*M* sodium [2-^{13}C]acetate or 300 m*M* [1-^{13}C]glucose, pH 7.4. The animals did not show any alteration of behavior after the injections. The animals were killed 5, 15, or 30 min after the injections. Another two groups of animals were injected subcutaneously. One group received a single subcutaneous bolus of 1 ml of 300 m*M* [2-^{13}C]acetate and was killed 30 min later. This was done to see whether subcutaneous and intravenous administration of [2-^{13}C]acetate yielded similar labeling of cerebral amino acids. Another group of animals was injected subcutaneously at 0, 10, and 20 min with 0.33 ml of 300 m*M* [2-^{13}C]acetate and killed at 30 min. This was done to establish a steady-state-like situation with respect to availability of [2-^{13}C]-acetyl-CoA in glia. In the steady state the C-3/C-4 labeling ratio in glutamine is an index of the percent loss of TCA cycle intermediates per turn of the cycle in glia (see below, as well as Hassel et al., 1994). Lastly, a group of animals was injected intravenously with 0.25 ml of 300 m*M* [1-^{13}C]glucose and killed after 2 min. In this group only the percent ^{13}C enrichment of blood glucose was measured.

The animals were killed by cervical dislocation, and the head and upper torso were immediately immersed in liquid nitrogen for 15 s. This caused the brain to freeze. The animals were then decapitated, and blood was collected and put on ice. The brains were removed and homogenized in 3 ml of 7% (vol/vol) perchloric acid with 50 m*M* NaH_2PO_4. In some cases brains were pooled two and two. The homogenates were centrifuged at 12,000 *g* for 20 min. The supernatants were neutralized with 9 *M* KOH. The precipitate, $KClO_4$, was removed by centrifugation (12,000 *g*, 20 min). Three hundred microliters (~10%) of the supernatant was taken for measurement of the total levels of amino acids, lactate, and glucose; the rest was lyophilized. The blood was used for glucose content determination; thereafter, protein was precipitated with 7% perchloric acid, and the supernatant was neutralized with KOH and lyophilized as described above for the brain extracts.

For ^{13}C NMR spectroscopy the lyophilisates were redissolved in 500 μl of D_2O with 0.5 μl of dioxane as internal standard. Inverse decoupled 125.7-MHz ^{13}C NMR spectra were obtained on a Bruker model AM-500 spectrometer. Spectra were accumulated using a 30° pulse angle and a spectral width of 31 KHz with 64K data points. The acquisition time was 1.049 s, and an additional relaxation delay of 3 s was used. Inversion recovery experiments were performed with the brain extracts to obtain relaxation values. Correction for saturation was not necessary for the resonances investigated because dioxane had a similar relaxation value and because the pulse angle was 30°. Furthermore, some of the samples were analyzed with relaxation delays of both 3 and 10 s without significant differences being found. For each spectrum typically 4,000 scans were accumulated. A line broadening of 1–5 Hz was used. Amino acids were analyzed by HPLC after derivatization with *o*-phthaldialdehyde, using α-amino adipic acid as internal standard (Paulsen et al., 1987). Glucose and lactate were analyzed by reflectance spectrophotometry using a DT 60 Ektachem (Kodak). Protein was analyzed spectrophotometrically according to the method of Lowry et al. (1951).

The percent enrichment with ^{13}C in a carbon position of a particular metabolite was calculated after subtraction of the naturally abundant ^{13}C (1.1%) as described by Badar-Goffer et al. (1990). In the brain extracts the percent ^{13}C enrichment of amino acids was measured: in blood extracts the percent ^{13}C enrichment of glucose was measured.

The percent loss of intermediates from the glial TCA cycle was derived from the C-3/C-4 labeling ratio in glutamine obtained with repeated subcutaneous injections of [2-^{13}C]-acetate (see Table 3). In the first turn of the TCA cycle [2-^{13}C]acetate yields labeling of the C-4 position in 2-oxoglutarate. This label is distributed equally between the C-2 and the C-3 positions in the next turn of the cycle owing to scrambling of the label in the symmetrical succinate step, and the label of the C-3 position is distributed equally between the C-2 and the C-3 positions in the subsequent turn of the cycle. The C-3 position therefore receives half the label in the C-4 and half the label in the C-3 position minus the fraction of intermediates lost per turn of the TCA cycle (Hassel et al., 1994). The C-3/C-4 labeling ratio can therefore be expressed as follows: $\text{C-3/C-4} = (\text{C-4} \times F/2 + \text{C-4} \times F^2/4 + \text{C-4} \times F^3/8 \cdots)/\text{C-4}$, which can be rewritten $\text{C-3/C-4} = \sum_{i=1}^{n} (F/2)^i$, from which the graph shown in Fig. 4 was drawn. C-3 and C-4 are the enrichments of the C-3 and C-4 positions of glutamine obtained with [2-^{13}C]acetate in the steady state (in the steady state C-4 is a constant value), n is the number of turns of the TCA cycle, and F is the fraction of TCA cycle intermediates that remains in the cycle per turn. The division by 2 comes from the scrambling of label in the symmetrical succinate step of the TCA cycle. A presupposition is that label does not enter the TCA cycle by an anaplerotic pathway, i.e., via pyruvate carboxylation. This would not occur to any significant extent with [2-^{13}C]acetate as the labeled precursor.

Calculation of ^{13}C enrichment ratios for the various carbon positions in the amino acids was performed for each individual animal to obtain the SE of the ratios.

Statistical analysis was performed with paired t test for differences in ^{13}C enrichment of carbon positions within the same molecule or between molecules in the same animal. When the data showed inhomogeneous variance, logarithmic transformation was performed before analysis. Statistical differences between treatment groups were analyzed with Student's t test.

RESULTS

The serum glucose level remained ~6 m*M* at all time points measured after intravenous injection of [2-^{13}C]acetate (data not shown). At 2, 5, 15, and 30 min after injection of [1-^{13}C]glucose, however, the serum

TABLE 1. *Total amounts of amino acids, lactate, and glucose in the brains of mice 15 min after intravenous injection with [1-^{13}C]glucose or [2-^{13}C]acetate*

Compound (n)	Concentration (nmol/mg of protein)						
	Glutamate	Glutamine	GABA	Aspartate	Alanine	Lactate	Glucose
[1-^{13}C]Glucose, i.v. (8)	84.2 ± 3.5[a]	37.6 ± 2.1	15.9 ± 0.9[a]	23.8 ± 1.2	3.8 ± 0.2	30.2 ± 3.0	29.7 ± 1.5
[2-^{13}C]Acetate, i.v. (7)	103.1 ± 2.2	43.6 ± 3.0	19.8 ± 0.6	24.1 ± 1.0	4.4 ± 0.2	34.5 ± 1.7	29.3 ± 0.2

Data are mean ± SEM values. The results obtained at 5 and 30 min after injection were very similar to those shown, which is why only the values at 15 min are shown.

[a] $p < 0.005$ by t test for difference from corresponding value obtained in animals injected intravenously with [2-^{13}C]acetate.

glucose level was 12.4 ± 0.7, 9.9 ± 0.2, 8.8 ± 0.5, and 7.6 ± 0.2 m*M*, respectively. After repeated subcutaneous injections of [2-^{13}C]acetate the serum glucose level was 7.2 ± 0.3 m*M* at 30 min. These values were all significantly higher ($p < 0.01$) than the serum glucose values after intravenous injection of [2-^{13}C]-acetate.

Injection of [2-^{13}C]acetate did not lead to detectable labeling of glucose in the serum at any time point whether administered intravenously or subcutaneously. Injection of [1-^{13}C]glucose, on the other hand, led to an enrichment of label at the C-1 in serum glucose of 57.4 ± 3.5% at 2 min (n = 5) and 22.1 ± 4.1% at 5 min (n = 5; mean ± SEM).

The levels of amino acids in the brain remained nearly constant from 5 to 30 min; therefore, only the values at 15 min are shown (Table 1). Glutamate and GABA levels were significantly higher in animals injected with [2-^{13}C]acetate compared with those injected with [1-^{13}C]glucose at 15 and 30 min. No significant differences were seen in the levels of the other amino acids, lactate, or glucose. It should be noted that although the serum glucose level was significantly higher in the glucose-injected animals, no differences were seen between animals injected with glucose or acetate in the cerebral levels of either glucose or lactate.

Labeling from [2-^{13}C]acetate injected intravenously

[2-^{13}C]Acetate labeled cerebral glutamate, aspartate, GABA, and glutamine but not glucose (Table 2 and Fig. 1; data for glucose not shown). The percent ^{13}C enrichment was higher in glutamine than in glutamate in all animals at all time points. The glutamine/glutamate relative enrichment ratios as calculated from the label enrichments of the C-4 positions in each animal were 4.7 ± 1.2, 2.1 ± 0.2, and 1.6 ± 0.07 at 5, 15, and 30 min after injection, respectively.

At 5 min after injection of [2-^{13}C]acetate glutamate and glutamine were labeled in the C-4 position only (Table 2). GABA was labeled primarily in the C-2 position (which corresponds to the C-4 position in glutamate and glutamine) and weakly in the C-3 and C-4 positions. The label enrichment of the C-3 position in GABA could not be reliably determined because of the interfering large [2-^{13}C]acetate peak. The C-2 and C-3 of aspartate were not significantly labeled at this time point.

At 15 min after injection of [2-^{13}C]acetate intravenously the ^{13}C enrichment had increased in the C-4 of glutamate and glutamine and in the C-2 of GABA, reflecting an increase in the formation of [2-^{13}C]-acetyl-CoA. In addition, the C-2 and the C-3 positions of glutamate and glutamine were labeled, showing cy-

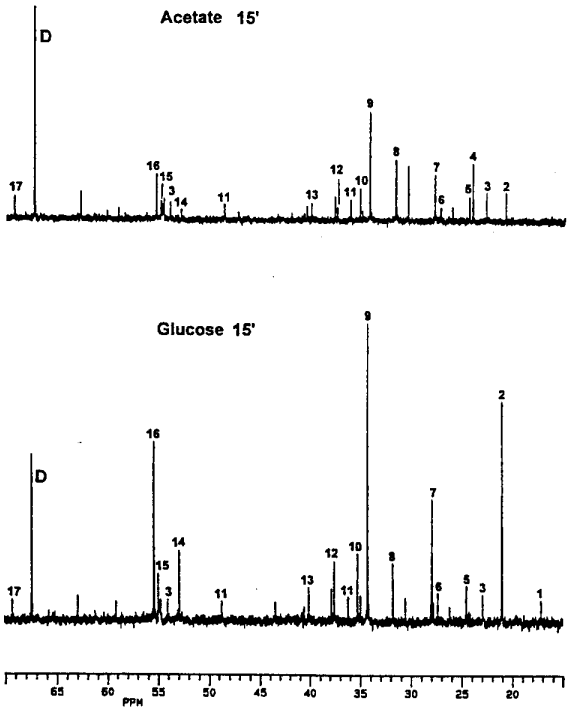

FIG. 1. ^{13}C NMR spectra of brain extracts from mice injected intravenously with [2-^{13}C]acetate (**upper panel**) or [1-^{13}C]-glucose (**lower panel**) with survival for 15 min. Peak assignment was as follows: 1, alanine C-3; 2, lactate C-3; 3, *N*-acetylaspartate; 4, acetate C-2; 5, GABA C-3; 6, glutamine C-3; 7, glutamate C-3; 8, glutamine C-4; 9, glutamate C-4; 10, GABA C-2; 11, taurine; 12, aspartate C-3; 13, GABA C-4; 14, aspartate C-2; 15, glutamine C-2; 16, glutamate C-2; 17, lactate C-2; and D, dioxane (internal standard).

TABLE 2. *Percent ^{13}C enrichments of the carbon positions of various amino acids after injection of [2-^{13}C]acetate*

Time (min), route	n	Glutamate			Glutamine			GABA			Aspartate	
		C-2	C-3	C-4	C-2	C-3	C-4	C-2	C-3	C-4	C-2	C-3
5, i.v.	7	ND	ND	0.67 ± 0.15	ND	ND	2.67 ± 0.51	1.20 ± 0.53	ND[a]	0.31 ± 0.05	ND	ND
15, i.v.	6	0.97 ± 0.17[b]	0.82 ± 0.15	2.78 ± 0.29	0.96 ± 0.29	0.97 ± 0.20	5.54 ± 0.44	3.85 ± 0.77	1.68 ± 0.55	1.02 ± 0.30	1.14 ± 0.27	2.00 ± 0.42
30, i.v.	6	2.37 ± 0.45[b]	1.34 ± 0.35	2.41 ± 0.46	2.68 ± 0.58	1.50 ± 0.78	3.78 ± 0.70	2.43 ± 0.41	2.97 ± 0.41[c]	2.21 ± 0.38	1.48 ± 0.26	1.47 ± 0.23
3×, s.c.	5	0.92 ± 0.09[b]	0.63 ± 0.12	1.94 ± 0.15	1.20 ± 0.17	1.04 ± 0.09	4.76 ± 0.29	2.02 ± 0.14	0.71 ± 0.19	1.15 ± 0.19[b]	1.28 ± 0.17	1.02 ± 0.23

Mice were injected intravenously with sodium [2-^{13}C]acetate (0.25 ml, 300 m*M*) and killed after 5, 15, or 30 min. Another group of animals was injected subcutaneously three times with 10-min intervals, with a 10-min survival after the last injection. Data are mean ± SEM values. ND, not detectable.

[a] This peak could not be reliably quantified because of the interfering peak of acetate C-2.

[b] $p < 0.05$ for difference from enrichment of the C-3 position in the same molecule.

[c] $p = 0.067$ by paired t test for difference from the enrichment of the C-4 position in the same molecule.

cling of label in the TCA cycle. In glutamate the C-2 position was more highly labeled than the C-3 position in all animals ($p = 0.02$ by paired t test), whereas no significant difference was found between the two positions in glutamine or between the corresponding C-4 and C-3 in GABA (Table 2). At this time point aspartate was labeled in the C-2 and C-3 positions with no significant difference between the two positions.

The C-3/C-4 labeling ratios in glutamate and glutamine (and the corresponding C-3/C-2 labeling ratio in GABA) reflect the number of times that labeled carbon skeletons have passed through the TCA cycle (see Materials and Methods). The C-3/C-4 labeling ratio at 15 min was 0.13 ± 0.02 in glutamine and 0.27 ± 0.05 in glutamate; the corresponding C-3/C-2 labeling ratio in GABA was 0.40 ± 0.01 (Table 3). Because the C-3 positions in glutamate and GABA are labeled through the TCA cycle, the significantly higher C-3/C-4 labeling ratios in glutamate and C-3/C-2 ratio in GABA indicate that some of the glutamine reentered and passed through TCA cycles before being converted to glutamate and GABA. On the other hand, some of the glutamine was converted directly to glutamate and GABA without involvement of the TCA cycle. This is evident from the labeling of the C-2 in GABA (Table 2), as the label in these positions is lost by passage through the TCA cycle (Figs. 2 and 3).

At 30 min after injection of [2-^{13}C]acetate, the percent enrichment had decreased in the positions that are labeled initially, i.e., the C-4 of glutamate and glutamine and the C-2 of GABA (Table 2). This shows that the entry of [2-^{13}C]acetyl-CoA into the TCA cycle was decreasing, which was in agreement with the absence of [2-^{13}C]acetate in the spectra (data not shown). On the other hand, the percent enrichment had increased in the positions that are labeled by the repeated passage of the carbon skeletons through the TCA cycle, i.e., the C-3 and the C-2 of glutamate and glutamine and the C-3 and the C-4 of GABA. This was also reflected in an increase in the C-3/C-4 labeling ratios in glutamate and glutamine and in the corresponding C-3/C-2 labeling ratio in GABA (Table 3).

TABLE 3. *The C-3/C-4 labeling ratio in glutamate (Glu) and glutamine (Gln) and the C-3/C-2 labeling ratio in GABA*

Precursor	Time (min)	n	Gln C-3/C-4	Glu C-3/C-4	GABA C-3/C-2
[2-^{13}C]Acetate, i.v.	15	6	0.13 ± 0.02[a]	0.27 ± 0.05[b]	0.40 ± 0.01[b]
[2-^{13}C]Acetate, i.v.	30	6	0.33 ± 0.14	0.55 ± 0.01	1.30 ± 0.20[b]
[2-^{13}C]Acetate, s.c.	30	3	0.32 ± 0.02[a]	0.58 ± 0.01[c]	0.43 ± 0.08
[2-^{13}C]Acetate, s.c., 10 min × 3	30	5	0.22 ± 0.02	0.31 ± 0.04	0.37 ± 0.12
[1-^{13}C]Glucose, i.v.	15	6	0.38 ± 0.10[d]	0.38 ± 0.02	0.41 ± 0.11
[1-^{13}C]Glucose, i.v.	30	5	0.61 ± 0.03	0.63 ± 0.09	0.47 ± 0.05

Mice were injected intravenously with [2-^{13}C]acetate or [1-^{13}C]glucose (0.25 ml, 300 m*M*) and killed after 15 or 30 min. Some mice were injected subcutaneously with [2-^{13}C]acetate either as a single bolus (1.0 ml) or three times (0.33 ml) with 10-min intervals (total survival time from first injection, 30 min). Data are mean ± SEM values. Intragroup differences were analyzed with paired two-tailed t test for each animal.

[a] $p < 0.05$, [c] $p < 0.01$ for difference from values obtained with repeated subcutaneous injection of [2-^{13}C]acetate.

[b] $p < 0.05$ for difference from corresponding ratio in Gln.

[d] $p < 0.05$ for difference from ratio in Gln obtained 15 min after intravenous injection of [2-^{13}C]acetate.

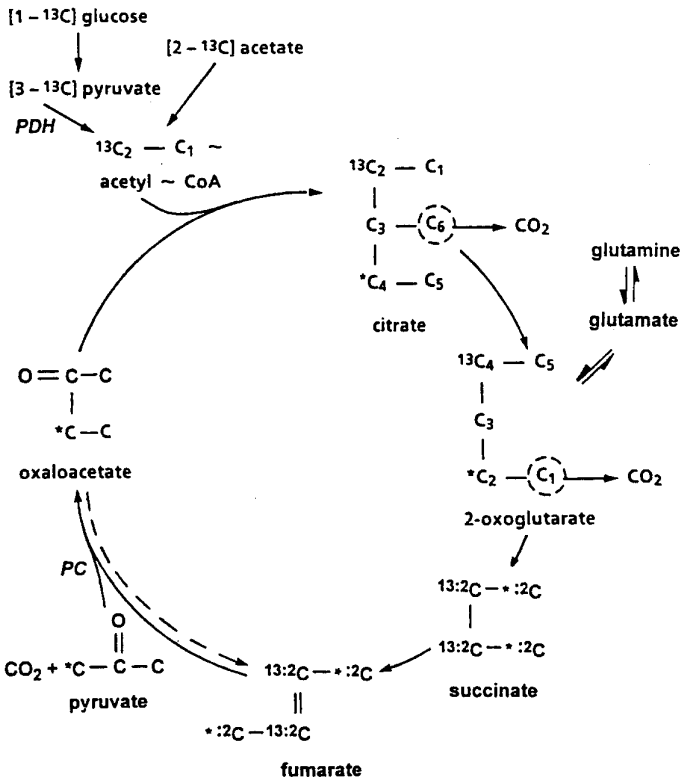

FIG. 2. Label distribution in TCA cycle intermediates from [1-^{13}C]glucose and [2-^{13}C]acetate. [1-^{13}C]-Glucose yields [3-^{13}C]pyruvate, which may yield [2-^{13}C]acetyl-CoA through pyruvate dehydrogenase (PDH) or, in glia, [3-^{13}C]oxaloacetate through pyruvate carboxylase (PC). In the first case 2-oxoglutarate (and hence glutamate and glutamine) is labeled in the C-4 position (^{13}C); in the latter case the label appears in the C-2 (*C). In glia [2-^{13}C]acetate yields [2-^{13}C]acetyl-CoA, which labels 2-oxoglutarate in the C-4. In the symmetrical succinate step the label is scrambled (symbolically $^{13:2}$C or $^{*:2}$C). The possible scrambling of label (*C) due to equilibration of oxaloacetate with fumarate is indicated by the broken arrow.

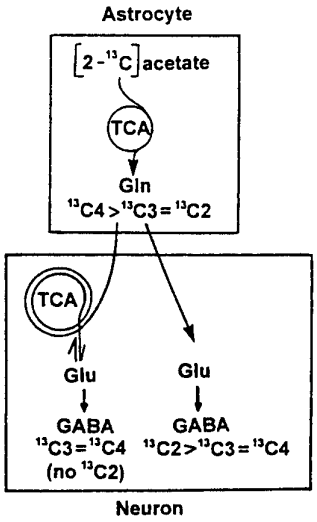

FIG. 3. Formation of GABA from [2-^{13}C]acetate. In glia [2-^{13}C]-acetate is converted to ^{13}C-labeled glutamine (Gln), which is transferred to GABAergic neurons. Here the Gln may be converted directly to GABA via glutamate (Glu), yielding GABA with a labeling of the C-2, C-3, and C-4 positions identical to the labeling of the C-4, C-3, and C-2 of Gln, respectively. Some of the Gln, however, is metabolized through the neuronal TCA cycle before being converted to Glu and GABA, leading to scrambling of the label in the C-4 of Gln between the C-3 and C-4 in GABA due to the symmetry of succinate (see Fig. 1).

The C-2 of glutamate remained more highly enriched than the C-3 even at 30 min, whereas there were no significant differences in ^{13}C enrichment between the C-2 and the C-3 positions in glutamine or aspartate 30 min after injection of [2-^{13}C]acetate. The C-3 of GABA, however, was more highly labeled than the C-4 in all animals, with marginal significance ($p = 0.067$ by paired t test).

Labeling from [2-^{13}C]acetate injected subcutaneously: determination of the loss of intermediates from the glial TCA cycle

At 30 min after a single subcutaneous injection of [2-^{13}C]acetate, the C-3/C-4 labeling ratios in glutamine and glutamate were 0.32 ± 0.02 and 0.58 ± 0.001, respectively (Table 3). This was quite similar to the values obtained 30 min after intravenous injection of [2-^{13}C]acetate (0.33 ± 0.14 and 0.55 ± 0.01; Table 3) and shows that values obtained after intravenous and subcutaneous injections of [2-^{13}C]acetate are comparable. [2-^{13}C]Acetate could not be seen in the spectra after 30 min whether given intravenously or subcutaneously, a sign that the C-3/C-4 labeling ratios in glutamine and glutamate were influenced by the lack of entry of new label into the TCA cycle. With repeated injections of [2-^{13}C]acetate the C-3/C-4 labeling ratio in glutamine was 0.22 ± 0.02 (Table 3). This value was lower than the ratio obtained 30 min after single intravenous or subcutaneous injection (difference from

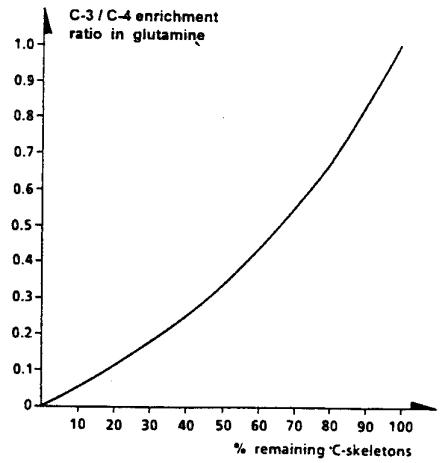

FIG. 4. C-3/C-4 enrichment ratio in glutamine as a function of the percentage of the TCA cycle intermediates (carbon skeletons) remaining in the glial TCA cycle per turn of the cycle during steady-state supply of [2-^{13}C]acetate (see Experimental animals). In the steady state the continuous loss of intermediates is the reason why the C-3/C-4 enrichment ratio does not approximate 1.

single subcutaneous injection, $p = 0.02$) but significantly higher than the ratio obtained 15 min after intravenous injection ($p = 0.02$), at which time the C-3/C-4 labeling ratios reflected an increasing supply of [2-^{13}C]acetyl-CoA (see above). The C-3/C-4 labeling ratio in glutamine obtained with repeated subcutaneous injections therefore fell between values that reflected either a rising or a falling concentration of acetyl-CoA. This supports the supposition that a steady-state-like situation was achieved with repeated subcutaneous injection of [2-^{13}C]acetate.

A C-3/C-4 labeling ratio of 0.22 in glutamine during steady state indicates that only 35% of the TCA cycle intermediates remains in the glial TCA cycle per turn (Fig. 4; Materials and Methods) and that 65% of the intermediates are lost per turn of the cycle.

Similar to the results obtained with intravenous injection, repeated subcutaneous injections of [2-^{13}C]-acetate led to a higher enrichment of the C-2 than of the C-3 position in glutamate (Table 2). Similarly, GABA was significantly more highly labeled in the C-4 than in the C-3 position, whereas in glutamine no significant difference could be found between the corresponding C-2 and C-3 positions.

Labeling from intravenous [1-^{13}C]glucose

Intravenous injection of [1-^{13}C]glucose led to ^{13}C enrichment in cerebral glutamate, GABA, glutamine, aspartate, and alanine (Table 4 and Fig. 1). The glutamine/glutamate relative labeling ratio calculated from the enrichments of the C-4 positions was 0.19 ± 0.04, 0.45 ± 0.04, and 0.50 ± 0.07 at 5, 15, and 30 min, respectively.

In contrast to what was seen with [2-^{13}C]acetate, glutamine was more highly labeled in the C-2 than in the C-3 position in all animals at 5 and 15 min after injection of [1-^{13}C]glucose. This reflects the activity of pyruvate carboxylase (EC 6.4.1.1), which labels the C-2 position initially (Fig. 2). The activity of pyruvate dehydrogenase (EC 1.2.4.1), on the other hand, leads to labeling of the C-4 position. This label is distributed equally between the C-2 and the C-3 positions through the TCA cycle (Fig. 2). By subtracting the C-3 labeling from the C-2 labeling in glutamine and dividing by the C-4 labeling, the labeling of glutamine through pyruvate carboxylase relative to that through pyruvate dehydrogenase can be determined. At 5 and 15 min, respectively, the flux of [3-^{13}C]pyruvate through pyruvate carboxylase was 58 ± 11 and 41 ± 5% of the flux through pyruvate dehydrogenase as determined from the labeling of glutamine (Table 5). At 30 min there was no difference between the enrichments of the C-2 and the C-3 positions in glutamine.

Glutamate was also labeled significantly better in the C-2 than in the C-3 position from [1-^{13}C]glucose at 5 and 15 min, but the contribution of pyruvate carboxylase to the labeling of glutamate was lower than for glutamine in all animals (Table 5), reflecting compartmentation of glutamate into glial and neuronal pools, of which only the former is associated with pyruvate carboxylation (see Discussion). At 30 min no significant difference in enrichment was found between the C-2 and C-3 in glutamate (Table 4).

There were no significant differences between the labeling of the C-3 and the C-4 positions of GABA at any time point after injection of [1-^{13}C]glucose (Table 4). At 5 min after injection, however, aspartate was more highly enriched in the C-2 than in the C-3 position ($p < 0.02$; Table 4). This was in contrast to what was expected, as pyruvate carboxylase activity would have given a higher enrichment of the C-3 than of the C-2 in oxaloacetate (Fig. 2).

The C-3/C-4 labeling ratios in glutamate and glutamine and the corresponding C-3/C-2 labeling ratio in GABA at 15 min were very similar, ~0.40 (Table 3). For glutamate and GABA these ratios were similar to the values obtained with [2-^{13}C]acetate, but the ratio in glutamine was significantly higher than the value obtained with [2-^{13}C]acetate at this time point (Table 3). This is compatible with equilibration of oxaloacetate with the symmetrical fumarate via malate, which would scramble the label (which enters the C-2 of oxaloacetate via pyruvate carboxylase) between the C-2 and the C-3 (see Discussion).

At 30 min after injection of [1-^{13}C]glucose the C-3/C-4 labeling ratios in glutamate and glutamine had increased somewhat (Table 3). This reflects a dwindling supply of [2-^{13}C]acetyl-CoA, as was evident from the absence of [1-^{13}C]glucose in the spectra at this time point.

The C-3 of alanine was strongly labeled from [1-^{13}C]glucose with a percent enrichment at 5, 15, and 30 min of 9.20 ± 0.58, 8.14 ± 0.91, and 5.55 ± 2.41,

TABLE 4. *Percent ^{13}C enrichments of the carbon positions of various amino acids in brain after injection of [1-^{13}C]glucose*

Time (min), route	n	Glutamate			Glutamine			GABA			Aspartate	
		C-2	C-3	C-4	C-2	C-3	C-4	C-2	C-3	C-4	C-2	C-3
5, i.v.	7	1.19 ± 0.12[a]	0.50 ± 0.11	5.43 ± 0.41	0.69 ± 0.13[a]	0.40 ± 0.11	1.05 ± 0.19	4.21 ± 0.50	0.76 ± 0.24	0.76 ± 0.18	4.06 ± 0.64[b]	2.60 ± 0.30
15, i.v.	6	3.44 ± 0.41[a]	2.29 ± 0.24	5.79 ± 0.48	1.85 ± 0.25[a]	0.94 ± 0.17	2.62 ± 0.37	6.54 ± 0.67	2.72 ± 0.54	3.19 ± 0.27	4.88 ± 0.31	4.89 ± 0.45
30, i.v.	5	3.44 ± 0.73	2.77 ± 0.44	4.47 ± 0.56	1.84 ± 0.55	1.65 ± 0.39	2.37 ± 0.54	6.13 ± 0.84	3.03 ± 0.64	3.11 ± 0.28	4.41 ± 0.84	4.30 ± 0.90

Mice were injected intravenously with [1-^{13}C]glucose (0.25 ml, 300 m*M*) and killed after 5, 15, or 30 min. Data are mean ± SEM values for the given number of animals (n).

[a] $p < 0.001$, [b] $p < 0.05$ by paired *t* test for difference from the C-3 position in the same molecule.

respectively. This reflects formation of [3-^{13}C]-pyruvate through glycolysis. The percent enrichment of the C-3 of alanine was significantly higher than the percent enrichment of the C-4 of glutamate at 5 and 15 min. This either suggests that alanine was labeled more rapidly than glutamate, e.g., because of postmortem glycolysis, or that a pool of glutamate was not readily accessible to labeling from [1-^{13}C]glucose (cf. Badar-Goffer et al., 1992).

DISCUSSION

Evaluation of the model

The interpretation of the data in the present report hinges on the assumption that acetate is metabolized in glia and not in neurons, whereas glucose is metabolized by both cell types but chiefly by neurons (see introductory section). Supporting this notion is the observation that the glutamine/glutamate relative enrichment ratio greatly exceeded 1 with [2-^{13}C]acetate as the precursor and was well below 1 with [1-^{13}C]-glucose as the precursor. These are characteristics of glial and neuronal metabolism, respectively (O'Neal and Koeppe, 1966; Berl, 1973; Hassel et al., 1992).

TABLE 5. *Relative labeling of glutamine and glutamate through pyruvate carboxylase versus pyruvate dehydrogenase from [1-^{13}C]glucose administered intravenously*

Time	n	Glutamine (C2 − C3)/C4	Glutamate (C2 − C3)/C4
5 min	7	0.58 ± 0.11	0.13 ± 0.00[a]
15 min	6	0.41 ± 0.05	0.19 ± 0.03[a]

Mice were injected intravenously with [1-^{13}C]glucose (0.25 ml, 300 m*M*) and were killed after 5 or 15 min. Data are mean ± SEM values, as the percent ^{13}C enrichment of the C-2 minus that of the C-3 divided by the enrichment of the C-4 in glutamine and glutamate in each animal.

[a] $p < 0.05$ by paired *t* test for difference from the corresponding value in glutamine.

Another important assumption is that extracerebral metabolism of the injected acetate and glucose did not contribute to the labeling patterns seen in brain metabolites. [2-^{13}C]Acetate did not lead to labeling of serum or brain glucose, which suggests that extracerebral conversion of acetate to glucose did not contribute significantly to the labeling of cerebral amino acids (cf. Cerdan et al., 1990). The contribution by extracerebral metabolism of labeled glucose to the labeling of cerebral metabolites has previously been shown to be minimal in fasted animals (Van den Berg, 1973) and in animals made hyperglycemic from injection of [1-^{13}C]glucose as in the present study (Mason et al., 1992).

The level of brain lactate found in this study suggests that anaerobic glycolysis took place before the tissue was frozen (cf. Pontén et al., 1973). This was probably not of any importance for the amino acid analysis, because previous studies have shown that the levels of amino acid remain constant 20–30 s after decapitation without freezing (see Siesjö, 1978). We therefore believe that our data on amino acids reflect the in vivo situation.

From the total amounts of cerebral amino acids in the brains of these animals it would seem that injection of acetate or glucose may affect the brain levels of glutamate and GABA. Additional experiments using the same doses of ^{12}C-acetate and ^{12}C-glucose confirmed these significant differences between the acetate- and the glucose-injected groups of animals, but none of the groups was significantly different from a group of uninjected animals (authors' unpublished data). More studies are required to clarify this issue.

Glial loss of TCA cycle intermediates and its replenishment through pyruvate carboxylation

We show in this study that astrocytes lose a major fraction of their TCA cycle intermediates. On average 65% of the intermediates were lost per turn of the TCA cycle as judged from the C-3/C-4 labeling ratios in glutamine obtained after repeated subcutaneous injec-

tions of [2-^{13}C]acetate. This is very similar to what was calculated for cultured astrocytes (Hassel et al., 1994). 2-Oxoglutarate, a precursor for glutamine, is the main TCA cycle intermediate to be lost from the glial TCA cycle. Other intermediates may be four-carbon skeletons, e.g., malate or oxaloacetate, that are decarboxylated to pyruvate (Hassel et al., 1994). This loss of TCA cycle intermediates has to be replenished by some anaplerotic activity lest the TCA cycle be drained. The main anaplerotic enzyme in brain is pyruvate carboxylase (Patel, 1974) (Fig. 2), which has a glial localization (Yu et al., 1983). The present results show a flux of pyruvate through pyruvate carboxylase of ~60% of the flux through the glial pyruvate dehydrogenase as calculated from the labeling of glutamine from [1-^{13}C]glucose 5 min after injection. The large flux through pyruvate carboxylase implies that astrocytes may replenish most if not all of the lost TCA cycle intermediates by this pathway. A flux of pyruvate through pyruvate carboxylase of 60% is higher than a recent finding (~40%) in the anesthetized rat during steady-state infusion of [1,2-$^{13}C_2$]glucose (Künnecke et al., 1993). However, as demonstrated by Shank et al. (1993), anesthesia may alter cerebral metabolism of [1-^{13}C]glucose. In the present study the labeling of glutamine after 15 min suggested that the flux through pyruvate carboxylase was indeed 40% of the flux through pyruvate dehydrogenase. At this time point, however, equilibration of oxaloacetate with the symmetrical fumarate could have led to scrambling of label between the C-2 and the C-3 positions as discussed by Berl and Frigyesi (1968) and by Van den Berg (1973). Subtracting the enrichment of the C-3 position from the C-2 in glutamine to assess the activity of pyruvate carboxylase would then yield too low a value.

It has previously been established that the metabolism of glucose through pyruvate carboxylase is ~10% of the total glucose metabolism (O'Neal and Koeppe, 1966; Van den Berg, 1973). Assuming the flux of pyruvate through pyruvate carboxylase to be 60% of that through the glial pyruvate dehydrogenase, the flux through the latter should account for 17% of the total brain glucose metabolism, and total glial metabolism of glucose could account for 27% of the total cerebral metabolism of glucose through the TCA cycle.

Formation of GABA from glutamine

The flux of glutamine from glia to neurons for the synthesis of GABA may be roughly estimated from the enrichment of GABA following injection of [2-^{13}C]acetate. Assuming that GABA is labeled from [2-^{13}C]acetate via glutamine (Van den Berg and Garfinkel, 1971; Cerdan et al., 1990; Sonnewald et al., 1993*b*), the amount of ^{13}C-labeled glutamine that has been converted to GABA can be calculated from the total amount of GABA multiplied by the percent enrichment of GABA (C-2 + C-3 + C-4), which at 5 min is 20 nmol/mg of protein × 1.5% = 0.3 nmol/mg of protein of ^{13}C-labeled glutamine (cf. Tables 1 and 2). This amount of [^{13}C]glutamine represents an enrichment of glutamine that corresponds to the enrichment of glutamine (C-2 + C-3 + C-4), which at 5 min is 2.7%, meaning that the total amount of glutamine transferred to GABAergic neurons in 5 min is 11 nmol/mg of protein, or 2.2 nmol/mg of protein/min (≈0.22 μmol/g of brain weight/min). This lies between the value of 0.14 μmol/g of brain weight/min calculated by Van den Berg and Garfinkel (1971) and the value of 0.36 μmol/g of brain weight/min calculated by Cremer et al. (1978).

The labeling of GABA from [2-^{13}C]acetate seen in this study probably occurs mainly via glutamine as suggested by previous studies (Van den Berg and Garfinkel, 1971; Cerdan et al., 1990; Sonnewald et al., 1993*b*). It is an interesting finding that the C-3/C-2 labeling ratio in GABA and the corresponding C-3/C-4 labeling ratio in glutamate are higher than the C-3/C-4 labeling ratio in glutamine when [2-^{13}C]acetate is the precursor (Table 3). This suggests that the carbon skeleton of glutamine is in part passed through TCA cycles before being converted to GABA and glutamate (Fig. 3). An implication of this finding is that glutamine is an energy substrate for neurons, as has been suggested from in vitro studies (Bradford et al., 1978; Yudkoff et al., 1989). Furthermore, this finding could imply that the neuronal TCA cycle is a control point in the synthesis of transmitter amino acids from glutamine. The actual fraction of the glutamine that is converted to GABA without being passed through TCA cycles can be calculated as follows: If GABA were formed directly from glutamine without the participation of the TCA cycle, the C-3/C-2 and C-4/C-2 enrichment ratios in GABA should equal the C-3/C-4 and the C-2/C-4 enrichment ratios in glutamine. (The enrichments of the C-3 and C-4 in GABA that yield ratios in excess of those in glutamine are due to passage of glutamine through the TCA cycle.) The C-3/C-4 and the C-2/C-4 enrichment ratios in glutamine after repeated subcutaneous injections of [2-^{13}C]acetate (from Table 2) are 0.22 and 0.25. The enrichment in the C-3 and C-4 of GABA that would yield corresponding ratios are 0.44 and 0.51%, respectively, because the enrichment of the C-2 of GABA is 2.02% (Table 2). The sum of these values plus the C-2 enrichment is 76% of the total GABA enrichment (2.02 + 0.71 + 1.15%; Table 2), which implies that 76% of the label in GABA has not been cycled in the TCA cycle of GABAergic neurons.

The present study does not demonstrate a contribution to the labeling of GABA from pyruvate carboxylation. This would have been seen as a higher enrichment of the C-4 than of the C-3 in GABA from [1-^{13}C]-glucose (Table 4). This may seem surprising as glutamine, the glial precursor for GABA, is highly labeled through pyruvate carboxylase (Cerdan et al., 1990; Shank et al., 1993; this study). GABA is, however, labeled both through neuronal metabolism of glucose and via glutamine from astrocytes, and the former path-

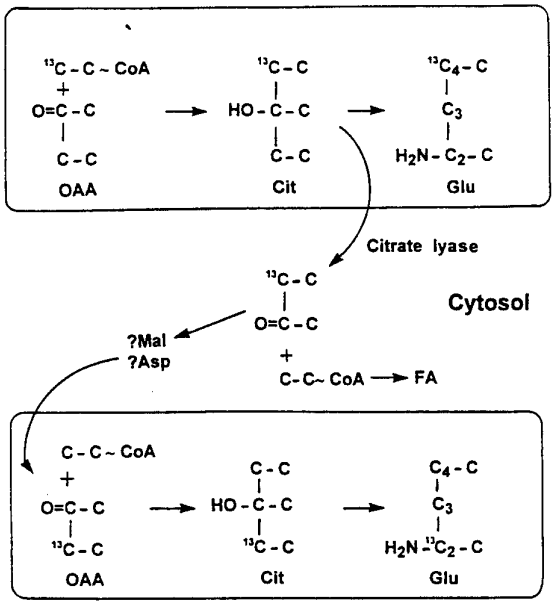

FIG. 5. The possible reactions leading to a higher ^{13}C enrichment of the C-2 than of the C-3 in glutamate (Glu) from [2-^{13}C]acetate: [2-^{13}C]Acetate is converted to [2-^{13}C]acetyl-CoA and [2-^{13}C]citrate ([2-^{13}C]Cit) in glia, which via the TCA cycle yields [4-^{13}C]Glu. With the next turn of the TCA cycle this label is distributed equally between the C-2 and C-3 in Glu (Fig. 1). [2-^{13}C]Cit may also leave the mitochondrion (rectangle) to undergo cleavage by Cit lyase in the cytosol, yielding [3-^{13}C]oxaloacetate ([3-^{13}C]OAA). This carbon skeleton may enter a mitochondrion, e.g., as malate (Mal) or aspartate (Asp), and will via the TCA cycle yield [4-^{13}C]Cit and hence [2-^{13}C]Glu. FA, fatty acids.

way probably overshadowed the latter in this study. In contrast to GABA, glutamate was significantly labeled through pyruvate carboxylation, but less than glutamine. This may be explained by the existence of more than one cerebral pool of glutamate, one that is the astrocytic precursor pool of glutamine and that is associated with pyruvate carboxylation (Yu et al., 1983) and other pools that are neuronal and not associated with pyruvate carboxylation.

Indication of an alternative pathway for isotopic labeling of glutamate and GABA

A peculiar labeling pattern obtained 15 min after intravenous injection and after repeated subcutaneous injections of [2-^{13}C]acetate is the higher enrichment of the C-2 than of the C-3 in glutamate. Similarly, GABA was more highly enriched in the C-4 than in the C-3 after repeated subcutaneous injections (Table 2). Had the two positions been labeled through the straightforward operation of the TCA cycle only, they would have had the same enrichment (Fig. 2). We offer the explanation that this labeling pattern reflects the initial step of fatty acid synthesis with cleavage of citrate to acetyl-CoA and oxaloacetate. [2-^{13}C]Citrate formed in glial mitochondria from [2-^{13}C]acetate may be transferred to the cytosol and cleaved by ATP citrate lyase (EC 4.1.3.28). This enzyme, which is active in brain (Patel, 1974), cleaves citrate between C-3 and C-4 (Schomburg and Salzmann, 1990), yielding [3-^{13}C]oxaloacetate from [2-^{13}C]citrate. If this carbon skeleton enters a TCA cycle, 2-oxoglutarate and hence glutamate would become labeled in the C-2 position (Fig. 5), and GABA would be labeled in the C-4 position. Van den Berg and Ronda (1976) have previously shown prominent labeling of lipids from [2-^{14}C]-acetate in mice, indicating a high activity of the citrate lyase pathway in glia.

At 30 min after intravenous injection of [2-^{13}C]-acetate the C-3 of GABA was more highly labeled than the C-4 (Table 2), which is the opposite of what is seen after repeated subcutaneous injections. At this time point the entry of [2-^{13}C]acetyl-CoA is dwindling, as discussed previously. Citrate will then chiefly be labeled in the C-3 and C-4 positions and would yield [2-^{13}C]oxaloacetate after cleavage by citrate lyase. This carbon skeleton would, after having entered a TCA cycle, yield 2-oxo[3-^{13}C]glutarate and hence [3-^{13}C]glutamate and [3-^{13}C]GABA.

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PAPER IV

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Glial Formation of Pyruvate and Lactate from TCA Cycle Intermediates: Implications for the Inactivation of Transmitter Amino Acids?

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Abstract: Cerebral formation of lactate via the tricarboxylic acid (TCA) cycle was investigated through the labeling of lactate from [2-^{13}C]acetate and [1-^{13}C]glucose as shown by ^{13}C NMR spectroscopy. In fasted mice that had received [2-^{13}C]acetate intravenously, brain lactate C-2 and C-3 were labeled at 5, 15, and 30 min, reflecting formation of pyruvate and hence lactate from TCA cycle intermediates. In contrast, [1-^{13}C]glucose strongly labeled lactate C-3, reflecting glycolysis, whereas lactate C-2 was weakly labeled only at 15 min. These data show that formation of pyruvate, and hence lactate, from TCA cycle intermediates took place predominantly in the acetate-metabolizing compartment, i.e., glia. The enrichment of total brain lactate from [2-^{13}C]acetate reached ~1% in both the C-2 and the C-3 position in fasted mice. It was calculated that this could account for 20% of the lactate formed in the glial compartment. In fasted mice, there was no significant difference between the labeling of lactate C-2 and C-3 from [2-^{13}C]acetate, whereas in fed mice, lactate C-3 was more highly labeled than the C-2, reflecting adaptive metabolic changes in glia in response to the nutritional state of the animal. It is hypothesized that conversion of TCA cycle intermediates into pyruvate and lactate may be operative in the glial metabolism of extracellular glutamate and GABA in vivo. Given the vasodilating effect of lactate on cerebral vessels, which are ensheathed by astrocytic processes, conversion of glutamate and GABA into lactate could be one mechanism mediating increases in cerebral blood flow during nervous activity. **Key Words:** Lactate—Glutamate—TCA cycle—Glia—Citrate lyase—^{13}C NMR spectroscopy.
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Cerebral formation of lactate is generally considered to reflect glycolysis whether anaerobic or aerobic. A small contribution, 1–3% of total lactate, is derived from the pentose phosphate shunt (Gaitonde et al., 1983; Hawkins et al., 1985; Ben-Yoseph et al., 1995). Recently, it was established, however, that astrocytes in culture release lactate formed via pyruvate from endogenous tricarboxylic acid (TCA) cycle intermediates (Hassel et al., 1994), and that cultured astrocytes convert exogenous glutamate to lactate via metabolism through the TCA cycle (Sonnewald et al., 1993*a*). The latter finding was repeated in brain slices (Bachelard et al., 1994). Balázs et al. (1970) reported radiolabeling of alanine from [1-^{14}C]GABA, which involves conversion of GABA into pyruvate. These studies suggested that inactivation of extracellular glutamate and GABA might involve conversion of these amino acids into pyruvate and hence lactate or alanine, and that this process could take place in glia. Moreover, lactate could be envisioned as having a role in the glial–neuronal trafficking of metabolites. These studies also shed light on the observation by Cremer and Heath (1974) that a pool of brain lactate appears to be derived neither from glycolysis nor from influx of serum lactate into brain. To address these issues, we studied whether pyruvate and lactate could be formed from TCA cycle intermediates in the intact brain, and, if so, in which cellular compartment this formation takes place.

^{13}C NMR in conjunction with administration of ^{13}C-labeled glucose and acetate has been used in the study of brain metabolism in vitro (Badar-Goffer et al., 1990, 1992; Sonnewald et al., 1991, 1993*b*; Hassel et al., 1994; Kauppinen et al., 1994) as well as in vivo both in animals (Behar et al., 1986; Cerdan et al., 1990; Fitzpatrick et al., 1990; Künnecke et al., 1993; Shank et al., 1993; Hassel et al., 1995) and in humans (Beckmann et al., 1991; Gruetter et al., 1994). [1-^{13}C]-Glucose metabolized through glycolysis may label lactate C-3. [2-^{13}C]Acetate, however, which cannot itself enter the glycolytic pathway, would not label lactate if lactate were formed via glycolysis alone. On the other hand, if pyruvate and hence lactate were formed

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Abbreviations used: i.v., intravenous; NMR, nuclear magnetic resonance; s.c., subcutaneous; TCA, tricarboxylic acid.

from TCA cycle intermediates, [2-^{13}C]acetate could, after conversion to acetyl-CoA and metabolism through the TCA cycle, label lactate. Similarly, [1-^{13}C]glucose metabolized through the TCA cycle could label lactate C-2, because of scrambling of label between CH_2 groups in the symmetrical succinate step of the TCA cycle (see Van den Berg, 1973; Hassel et al., 1994).

Acetate is generally believed to be metabolized by astrocytes and not by neurons, whereas glucose is metabolized by both cell types, but chiefly by neurons (O'Neal and Koeppe, 1966; Van den Berg, 1973; Minchin and Beart, 1975; Muir et al., 1986; Hassel et al., 1992, 1995; Sonnewald et al., 1993*b*). Therefore, the labeling of lactate from these precursors may indicate the cell type in which the formation of lactate from TCA cycle intermediates occurs.

MATERIALS AND METHODS

Materials

Female NMRI mice, 20–30 g, were obtained from Bomholt (Ry, Denmark). Animals were caged in groups of 10 at 21°C and 50% humidity, with a 12-h light/dark cycle and free access to food and tap water. Sodium [2-^{13}C]acetate and [1-^{13}C]glucose were obtained from Sigma (St. Louis, MO, U.S.A.). Before injection, the ^{13}C-labeled compounds were dissolved in double-distilled water to a concentration of 300 m*M*, and pH was adjusted to 7.4 with weak HCl or NaOH.

Experimental procedures

Mice were fasted overnight with free access to tap water. Without prior anesthesia, 0.25 ml of a solution of sodium [2-^{13}C]acetate or [1-^{13}C]glucose was injected into a tail vein over 5 s. At 2, 5, 15, or 30 min, the animals were killed by cervical dislocation and the heads were immersed immediately in liquid nitrogen for 15 s, which caused the brains to freeze. The animals were then decapitated, and blood was collected from the severed vessels and put on ice. Serum was separated by centrifugation and analyzed for glucose and lactate. To achieve a steady-state-like supply of [2-^{13}C]acetate (Hassel et al., 1995), one group of animals received 0.33 ml of the [2-^{13}C]acetate solution subcutaneously (s.c.) at 0, 10, and 20 min, and was killed at 30 min. This procedure also allowed us to study the effect of intravenous (i.v.) administration of [2-^{13}C]acetate (s.c. versus i.v.), and to see to what extent metabolism of [2-^{13}C]acetate in fat tissue would influence lactate labeling in the brain extracts. To assess the influence of the nutritional state upon cerebral formation of lactate from TCA cycle intermediates, a group of fed mice received sodium [2-^{13}C]acetate i.v. and was killed at 15 min.

Brains were homogenized in 3 ml of 7% ice-cold perchloric acid with 50 m*M* NaH_2PO_4. Protein was removed by centrifugation, and the supernatant was neutralized with 9 *M* KOH. The precipitate, $KClO_4$, was removed by centrifugation. The supernatant was lyophilized to dryness and redissolved in 0.5 ml of D_2O with 0.5 μl of dioxane as internal standard. Extraction of serum was done with 2 ml of 7% perchloric acid/0.5 ml of serum. The supernatant was neutralized with 9 *M* KOH, and was processed further in the same way as the brain extracts. Proton-decoupled 125.7-MHz ^{13}C NMR spectra were obtained on a Bruker AM-500 spectrometer. Approximately 4,000 scans were accumulated per spectrum using a 30° pulse angle and a line broadening of 1–5 Hz. Acquisition time was 1.049 s. Inverse gating with a delay time of 3 s was used to minimize nuclear Overhauser effects. Assignments were made on the basis of previous reports (Bárány et al., 1985; London, 1988). The total level of acetate in serum extracts was quantified by ^{1}H NMR spectroscopy; 500-MHz ^{1}H NMR spectra were accumulated using a 40° pulse angle, 15-s relaxation delay, and a sweep width of 5 kHz. A line broadening of 0.3 Hz was applied. Sixty-four scans were accumulated per ^{1}H NMR spectrum. All spectra were recorded at ambient temperature. Inversion recovery experiments were performed in order to obtain relaxation (T1) values both for ^{1}H and ^{13}C NMR. Correction for saturation was not necessary for the resonances investigated. The total levels of glucose and lactate in serum and in extracts of brain and serum were measured by reflectance spectrophotometry using a DT 60 Ektachem (Kodak). Amino acids were analyzed by HPLC after derivatization with *o*-phthaldialdehyde (Paulsen et al., 1987). The protein pellet was dissolved in 0.1 *M* NaOH with 0.2% sodium dodecyl sulfate and measured according to Lowry et al. (1951). The ^{13}C enrichment of metabolites was calculated after subtraction of the naturally abundant ^{13}C (1.1% of total amount of carbon) (Badar-Goffer et al., 1990). The peaks of malate C-3 and succinate C-2 + C-3 in the ^{13}C NMR spectra were too small to be quantified reliably. Moreover, malate and succinate were not visible in the ^{1}H NMR spectra. Therefore, the percentage enrichment of these compounds is not given.

The statistical significance of the values for ^{13}C labeling of lactate from [2-^{13}C]acetate was assessed by the Student's *t* test. Differences in ^{13}C enrichment between lactate C-2 and C-3 after injection of [2-^{13}C]acetate were analyzed for each animal using the paired *t* test.

RESULTS

In fasted mice, injection of [2-^{13}C]acetate i.v. led to significant labeling of lactate C-2 and C-3 in brain extracts at both 15 (difference from zero, $p < 0.05$, *t* test) and 30 min ($p < 0.02$) (Table 1; Fig. 1). The level of enrichment was approximately 1%, and there was no significant difference between the two positions with respect to ^{13}C enrichment at any of the two time points. At 5 min after injection of [2-^{13}C]acetate, all animals showed ^{13}C enrichment of lactate C-2 ($p = 0.02$), whereas lactate C-3 was detectably enriched only in three of six animals. Repeated s.c. injection of [2-^{13}C]acetate also led to enrichment of ~1% in lactate C-2 and C-3 ($p < 0.05$), with no significant difference in the labeling of the two positions. In fed mice, on the other hand, [2-^{13}C]acetate yielded a significantly higher enrichment of lactate C-3 than of C-2 (Table 1). [1-^{13}C]Glucose labeled lactate C-3 in brain extracts at all time points examined, whereas lactate C-2 was weakly, but significantly, labeled only at 15 min after injection ($p < 0.01$) (Table 1).

Injection of [1-^{13}C]glucose led to larger peaks of ^{13}C-labeled succinate and malate in the ^{13}C NMR spectra at 5, 15, and 30 min than did injection of [2-^{13}C]-

TABLE 1. *Percentage of ^{13}C enrichment of brain lactate C-2 and C-3 and glucose after injection of [2-^{13}C]acetate or [1-^{13}C]glucose*

	[2-^{13}C]Acetate				[1-^{13}C]Glucose			
Min and route	n	Lactate C-2	Lactate C-3	Glucose	n	Lactate C-2	Lactate C-3	Glucose C-1
2 min (i.v.)	6	ND	ND	ND	5	ND	15.03 ± 1.66	25.32 ± 3.32
5 min (i.v.)	6	0.60 ± 0.25	NS	ND	8	ND	17.57 ± 1.60	9.82 ± 2.38
15 min (i.v.)	5	1.56 ± 0.73	1.01 ± 0.35	ND	6	0.20 ± 0.07	10.77 ± 0.99	4.81 ± 0.45
30 min (i.v.)	5	0.68 ± 0.22	1.30 ± 0.33	ND	5	ND	7.79 ± 1.79	ND
10 × 3 min (s.c.)[a]	5	1.19 ± 0.32	1.00 ± 0.32	ND				
15 min (i.v.)[b]	5	0.22 ± 0.09	1.70 ± 0.38[c]	ND				

Sodium [2-^{13}C]acetate or [1-^{13}C]glucose (0.25 ml, 0.3 *M*) was injected i.v. into awake mice, which were killed at 2, 5, 15, or 30 min. Mice were fasted for 20 h prior to experiments. Data represent percentages of ^{13}C enrichment (means ± SEM). All values are significantly different from zero at $p < 0.05$. ND, not detectable. NS, not significant. ^{13}C enrichment was detectable only in three of six animals.

[a] Sodium [2-^{13}C]acetate was injected s.c. at 0, 10, and 20 min, and animals were killed at 30 min.

[b] Mice in this group were fed, not fasted.

[c] Different from corresponding value in lactate C-2; $p < 0.05$, paired *t* test.

acetate (Fig. 1). These peaks were absent in the brains of fed mice that received [2-^{13}C]acetate (Fig. 1).

Two minutes after injection of [1-^{13}C]glucose, significant ^{13}C enrichment was found in glutamate C-4 and GABA C-2 (which corresponds to glutamate C-4), whereas glutamine was not detectably labeled at this time point (Table 2), confirming the predominantly neuronal metabolism of [1-^{13}C]glucose (see Discussion). In contrast, 2 min after injection of [2-^{13}C]acetate, glutamine was more highly enriched than glutamate (Table 2). This labeling pattern, which is indicative of glial metabolism (see Discussion), was also seen in fed mice killed 15 min after injection of [2-^{13}C]acetate (Table 2).

The total brain level of lactate remained constant at all time points investigated (2, 5, 15, and 30 min), and did not differ significantly between the acetate- and glucose-injected animals. Thus, at 2 min after injection of [2-^{13}C]acetate, brain lactate was 33.4 ± 1.41 nmol/mg of protein, and 2 min after injection of [1-^{13}C]glucose, brain lactate was 31.6 ± 3.10 nmol/mg of protein. Likewise, in fed mice given [2-^{13}C]acetate and killed at 15 min, brain lactate was 32.5 ± 2.11 nmol/mg of protein.

The ^{13}C enrichment of serum lactate after injection of [2-^{13}C]acetate and [1-^{13}C]glucose was examined to determine whether the enrichment of brain lactate could have been influenced by the blood trapped in cerebral vessels. In fed mice that received [2-^{13}C]-acetate, serum lactate was not detectably labeled in either the C-2 or the C-3, which was in contrast to the labeling of brain lactate in these animals. Repeated s.c. injections into fasted mice led to detectable labeling of serum lactate C-3, but not C-2, a labeling pattern that was quite different from that seen in brain lactate. In the serum extracts of these animals, a peak corresponding to citrate C-2 appeared, but none that could have corresponded to citrate C-3. In fasted animals given [2-^{13}C]acetate i.v., on the other hand, serum lactate was labeled both in the C-2 and in the C-3 positions at all time points (Table 3), but no other metabolites were seen to be labeled. [1-^{13}C]Glucose labeled serum lactate C-3 at all examined time points, whereas the C-2 position remained unlabeled (Table 3).

At 2, 5, 15, and 30 min after injection of [2-^{13}C]-acetate, serum lactate was 9.9 ± 0.4, 4.3 ± 0.2, 3.8 ± 0.6, and 2.7 ± 0.6 m*M*., respectively (means ± SEM, n = 4–6, see Table 3). In fed mice that received [2-^{13}C]acetate, serum lactate was 6.0 ± 0.6 m*M* at 15 min (n = 5). (See Discussion for calculation of the contribution of ^{13}C-labeled serum lactate to the values in brain extracts.)

The percentage enrichment of serum acetate and glucose was calculated after injection of [2-^{13}C]acetate and [1-^{13}C]glucose, respectively. This was done to examine whether differences in ^{13}C enrichment of serum acetate and glucose could explain the differences seen in the ^{13}C labeling of brain lactate. Two minutes after injection of [2-^{13}C]acetate, the ^{13}C enrichment of serum acetate C-2 was 77.0 ± 2.0%; 2 min after injection of [1-^{13}C]glucose, the ^{13}C enrichment of serum glucose C-1 was 51.9 ± 6.2%.

DISCUSSION

The present study shows labeling of lactate from [2-^{13}C]acetate in both brain and serum. This lactate must have been formed from pyruvate originating from TCA cycle intermediates because acetate cannot itself enter the glycolytic pathway, nor the pentose phosphate pathway. By conversion of acetate into acetyl-CoA and metabolism through the TCA cycle, however, the label is incorporated into malate and oxaloacetate, which may be decarboxylated to pyruvate (Frenkel, 1972; Clarke and Berl, 1973). The much higher percentage enrichment of glutamine than of glutamate from [2-^{13}C]acetate at 2 min agrees with metabolism of acetate in the glutamine-synthesizing compartment, which is glia (Martinez-Hernandez et al., 1977). Labeling of

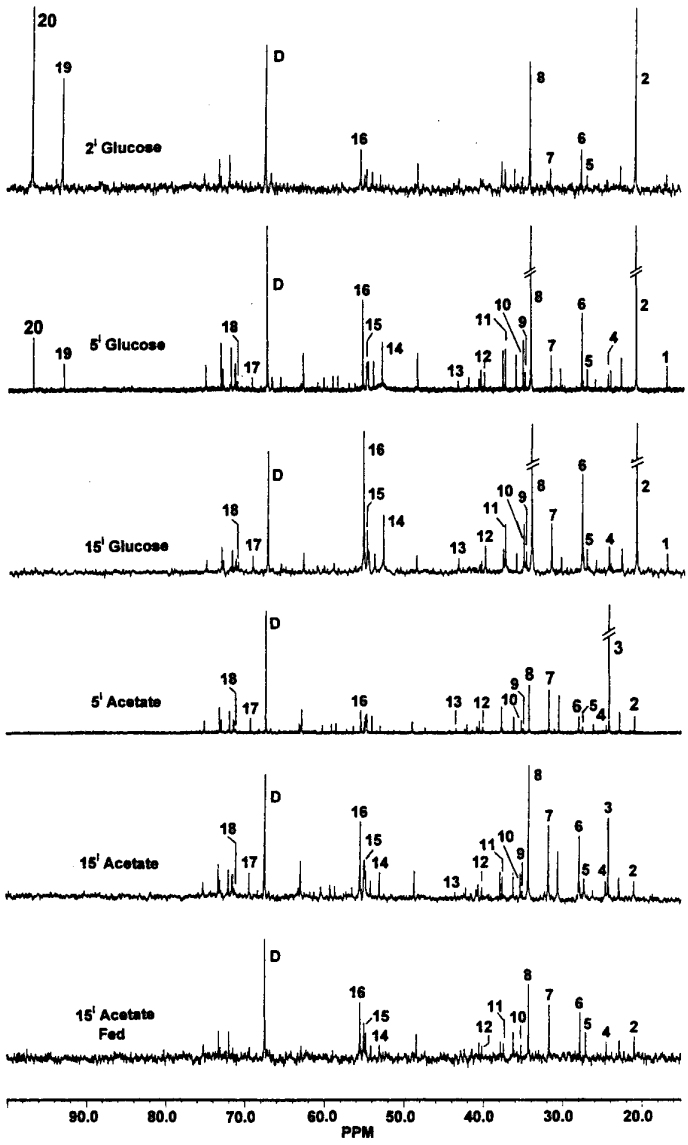

FIG. 1. Typical ^{13}C NMR spectra of extracts of mouse brain. Brains were removed 2, 5, and 15 min after i.v. injection of [1-^{13}C]glucose and 5 and 15 min after i.v. injection of sodium [2-^{13}C]acetate. The animals were fasted for 20 h prior to experiments, except for one of the groups, which received [2-^{13}C]acetate (lower spectrum). Peaks identified by numbers: 1, alanine C-2; 2, lactate C-3; 3, acetate C-2; 4, GABA C-3; 5, glutamine C-3; 6, glutamate C-3; 7, glutamine C-4; 8, glutamate C-4; 9, succinate C-2 + C-3; 10, GABA C-2; 11, aspartate C-3; 12, GABA C-4; 13, malate C-3; 14, aspartate C-2; 15, glutamine C-2; 16, glutamate C-2; 17, lactate C-2; 18, malate C-2; 19 and 20, the α and β anomers of glucose C-1, representing the two rotational states of glucose C-1.

glutamate and GABA, but not of glutamine, from [1-^{13}C]glucose at 2 min agrees with a predominantly neuronal metabolism of glucose (O'Neal and Koeppe, 1966; Berl, 1973; Minchin and Beart, 1975; Hassel et al., 1992, 1995). The finding that [2-^{13}C]acetate is a better precursor for C-2- and C-3-labeled lactate than [1-^{13}C]glucose is for C-2-labeled lactate therefore indicates that the formation of pyruvate from TCA cycle intermediates took place predominantly in the acetate-metabolizing compartment, i.e., glia. Conversion of TCA cycle intermediates into pyruvate has been demonstrated previously in brain slices with the use of [2-^{14}C]acetate (Cheng and Nakamura, 1972), [1-^{14}C]-GABA (Balázs et al., 1970), and [U-^{13}C]glutamate (Bachelard et al., 1994). These experiments show formation of pyruvate from TCA cycle intermediates in brain tissue in vitro. However, the cellular compartment in which the conversion took place was not addressed in those studies.

The total formation of lactate from TCA cycle intermediates may be calculated as follows. With repeated s.c. injection of [2-^{13}C]acetate, the enrichment of lactate C-2 + C-3 was ~2%. Under the same conditions, the enrichment of glutamate C-3 + C-4 from [2-^{13}C]-acetate was ~2.6% (Hassel et al., 1995). The glutamate labeled from [2-^{13}C]acetate mainly represents glial glutamate (Van den Berg and Van den Velden, 1970; Van den Berg, 1973), which has a metabolic half-life of ~1 min (Fitzpatrick et al., 1990). With repeated s.c. injections of [2-^{13}C]acetate, this pool has

TABLE 2. *Percentage of ^{13}C enrichment of cerebral amino acids from [1-^{13}C]glucose and [2-^{13}C]acetate*

		Glutamate			Glutamine			GABA		
Precursor	n	C-2	C-3	C-4	C-2	C-3	C-4	C-2	C-3	C-4
[1-^{13}C]Glucose, 2 min	6	ND	ND	1.46 ± 0.24	ND	ND	ND	1.14 ± 0.18	ND	ND
[2-^{13}C]Acetate, 2 min	6	0.42 ± 0.14	0.31 ± 0.12	1.40 ± 0.12	0.99 ± 0.24	0.54 ± 0.11	4.94 ± 0.25	ND	ND	ND
[2-^{13}C]Acetate, 15 min (fed)	5	1.48 ± 0.19	0.73 ± 0.09	2.01 ± 0.14	2.72 ± 0.32	1.33 ± 0.25	5.02 ± 0.61	2.85 ± 0.73	1.93 ± 0.47	1.99 ± 0.17

[1-^{13}C]Glucose or sodium [2-^{13}C]acetate (0.25 ml, 300 m*M*) was injected i.v. into fasted mice, which were killed at 2 min (upper two rows). The lower row presents data for fed mice that received sodium [2-^{13}C]acetate i.v. and were killed at 15 min. Values are means ± SEM. ND, not detectable.

a steady-state enrichment (see Hassel et al., 1995). As this glutamate is approximately 10% of the total pool (Cooper et al., 1988), its labeling is diluted 1:10 by unlabeled neuronal glutamate. Therefore, the actual enrichment of glial glutamate is ~26%. Because there is an equilibrium between glutamate and 2-oxoglutarate (Van den Berg, 1973; Mason et al., 1992), the enrichment of the C-3 + C-4 of glial 2-oxoglutarate is also 26%. If this 2-oxoglutarate and the lactate formed from TCA cycle intermediates were derived from the same (glial) TCA cycle, these two metabolites should have the same percentage enrichment. The label in the C-3 and C-4 of 2-oxoglutarate is scrambled between the C-2 and C-3 of succinate. Therefore, the C-2 + C-3 of glial malate, oxaloacetate, and the lactate formed from them, would have an enrichment of 26%. The observed enrichment of the C-2 + C-3 of lactate is 2%, however, which means that this lactate is diluted 1:13 by unlabeled, glycolytic lactate. Consequently, 7–8% of the lactate formed in these animals could be from glial TCA cycle intermediates. (This percentage may in fact be even greater because GABA is taken up by glial cells and converted to succinate, which would dilute the label in glial succinate.) Ninety percent of cerebral lactate may therefore be formed by glycolysis, the rest being formed via the pentose phosphate pathway (1–3%, see above) and from glial TCA cycle intermediates. As glia metabolize approximately 30% of cerebral glucose (Giacobini, 1964; Hassel et al., 1995), 30% of the lactate formed by glycolysis may be assumed to be glial, that is 27% of total lactate. The lactate formed from TCA cycle intermediates (~7% of total lactate) could therefore account for 20% of the total glial pool of lactate. The percent enrichment of lactate from [2-^{13}C]acetate is in itself not a measure of the pool size of the lactate formed via this pathway.

In the fed animals, serum lactate and serum glucose remained completely unlabeled by [2-^{13}C]acetate, which shows that the labeled lactate in brain extracts was formed in the brain itself without contamination from blood. Further, no other labeled metabolites were seen in serum extracts, which could have served as precursors for brain lactate. In animals that received repeated s.c. injections, brain lactate was labeled equally well in the C-2 and C-3 positions, whereas serum lactate was labeled in the C-3 only. As shown by O'Neal and Koeppe (1966), lactate that enters the brain from the bloodstream is metabolized in the large, neuronal compartment, as is glucose. The C-3-labeled serum lactate will therefore be an equally poor precursor of C-2-labeled brain lactate as [1-^{13}C]glucose. In these groups of animals, therefore, extracerebral metabolism of [2-^{13}C]acetate cannot explain the labeling of lactate seen in the brain extracts.

In the fasted animals that received [2-^{13}C]acetate i.v., some possible sources of error need to be considered. The ^{13}C enrichment of serum lactate in these animals was quite high and must have contributed

TABLE 3. *Percentage of ^{13}C enrichment of the C-2 and C-3 in serum lactate after injection of [2-^{13}C]acetate or [1-^{13}C]glucose*

		[2-^{13}C]Acetate			[1-^{13}C]Glucose	
Min and route	n	Lactate C-2	Lactate C-3	n	Lactate C-2	Lactate C-3
2 min (i.v.)	6	0.84 ± 0.23	1.50 ± 0.17	5	ND	4.68 ± 0.50
5 min (i.v.)	6	2.91 ± 1.15	5.79 ± 0.89	8	ND	7.76 ± 0.99
15 min (i.v.)	5	3.38 ± 1.05	4.31 ± 1.65	4	ND	6.43 ± 0.86
30 min (i.v.)	4	5.57 ± 1.15	4.93 ± 0.76	3	ND	6.67 ± 1.40
10 × 3 min (s.c.)[a]	5	ND	4.10 ± 1.65			
15 min (i.v.)[b]	5	ND	ND			

Sodium [2-^{13}C]acetate or [1-^{13}C]glucose (0.25 ml, 0.3 *M*) was injected i.v. into awake mice, which were killed at 2, 5, 15, or 30 min by cervical dislocation and immersion in liquid nitrogen, after which blood was collected. Mice were fasted for 20 h prior to experiments. Data represent percentages (means ± SEM). ND, not detectable.

[a] Sodium [2-^{13}C]acetate was injected s.c. at 0, 10, and 20 min, and animals were killed at 30 min.

[b] Mice in this group were fed, not fasted.

somewhat to the labeling of the lactate in brain extracts. At 15 min, the concentration of serum [2-^{13}C]-lactate was ~0.14 m*M* (serum lactate was ~4 m*M* and the ^{13}C enrichment of serum lactate C-2 was ~3.4%). Cerebral blood volume in mice corresponds at most to 0.4 μl of blood/mg of brain protein (Edvinsson et al., 1973). This means that in the brain extracts, serum [2-^{13}C]lactate accounted for 0.05 nmol/mg of protein. The total amount of [2-^{13}C]lactate in the brain extracts at 15 min was 0.47 nmol/mg of protein (total brain lactate was ~30 nmol/mg of protein with 1.56% enrichment). Therefore, at this time point, ~11% of the lactate in the brain extracts was from blood trapped in the cerebral vessels.

Cerebral uptake of labeled lactate from serum may also have taken place in fasted animals. It has been estimated previously that the uptake of lactate and pyruvate from serum amounts to 0.50 nmol/mg of protein/min (Pardridge and Oldendorf, 1977). From five to 15 min, the enrichment of lactate C-2 remained fairly constant, around 3% (Table 3), meaning that 0.015 nmol/mg of protein of [2-^{13}C]lactate could have entered the brain per minute. With a pool size of lactate of 30 nmol/mg of protein and a metabolic rate corresponding to 16 nmol of lactate/mg of protein/min (Mason et al., 1992), an ~2-min delay from the uptake of lactate from the bloodstream until its further metabolism can be estimated. In 2 min, 0.03 nmol/mg of protein of [2-^{13}C]lactate may have entered the brain. Together with the serum [2-^{13}C]lactate of the cerebral blood volume calculated above (0.05 nmol/mg of protein), this blood-derived [2-^{13}C]lactate could account for ~17% of the [2-^{13}C]lactate seen in the brain extracts at 15 min. We therefore conclude that most of the [2-^{13}C]lactate in the brain extracts was of cerebral origin, even in these animals.

Serum acetate had an enrichment of 77.0% 2 min after injection of [2-^{13}C]acetate, whereas serum glucose had an enrichment of 51.9% 2 min after injection of [1-^{13}C]glucose. This difference cannot explain the much higher ^{13}C enrichment of brain lactate C-2 from [2-^{13}C]acetate than from [1-^{13}C]glucose. Further, the peaks representing TCA cycle intermediates succinate and malate in the ^{13}C NMR spectra became higher with [1-^{13}C]glucose than with [2-^{13}C]acetate. Therefore, the better labeling of lactate C-2 from [2-^{13}C]acetate than from [1-^{13}C]glucose in fasted animals cannot be explained by differences in enrichment of the precursors of C-2-labeled lactate.

An unexpected finding of this study was the marked difference in the way fed and fasted mice metabolized [2-^{13}C]acetate. In fasted mice, [2-^{13}C]acetate labeled brain lactate with no significant differences between the C-2 and C-3 positions. In fed animals, however, [2-^{13}C]acetate labeled mainly brain lactate C-3. The first step in the metabolism of [2-^{13}C]acetate to [^{13}C]-lactate is the formation of C-2-labeled citrate. Citrate may be metabolized through the TCA cycle to yield malate or oxaloacetate, and in each the ^{13}C will be distributed equally between the C-2 and the C-3 positions because of scrambling of label in the symmetrical succinate step. Pyruvate, and hence lactate, formed after decarboxylation of ^{13}C-labeled malate or oxaloacetate, will accordingly be labeled equally in the C-2 and the C-3 positions. This pathway may fully account for the accumulation of label in the fasted, but not in the fed mice. In fed mice, an alternative pathway would be the transfer of citrate to the cytosol with subsequent cleavage by ATP citrate lyase (EC 4.1.3.28), which cleaves citrate between the C-3 and the C-4 position (Schomburg and Salzmann, 1990), forming oxaloacetate labeled in the C-3. Decarboxylation of oxaloacetate (or malate) will yield pyruvate and hence lactate labeled only in the C-3. It is interesting that both pyruvate carboxylase, which decarboxylates oxaloacetate (Clarke and Berl, 1973), and cytosolic malic enzyme, which decarboxylates malate (Frenkel, 1972), are located in glia and not in neurons (Yu et al., 1983; Kurz et al., 1993*a*,*b*). It seems, therefore, that in the brains of fasted animals glial citrate was metabolized mainly through the TCA cycle, whereas in the fed animals a high proportion of the citrate was exported to the cytosol to undergo cleavage by ATP citrate lyase, which is the initial step of fatty acid synthesis. This would be compatible with a preferred use of citrate as an energy source during fasting, whereas in the fed state anabolic lipogenesis is allowed. In this context it should be noted that the labeling of brain lactate C-3 from [1-^{13}C]glucose may represent both glycolysis and cleavage of citrate labeled in the C-2 from [1-^{13}C]glucose. Even the strong labeling of serum lactate C-3 from [1-^{13}C]glucose may to some degree reflect citrate cleavage. Cleavage of citrate labeled from [^{14}C]glucose has been shown to be active in both the liver and fat tissue of the rat (Ballard and Hanson, 1967; Hanson and Ballard, 1967). The labeling of serum lactate C-3 from [2-^{13}C]acetate injected repeatedly into the subcutaneous fat tissue agrees with the high activity of citrate cleavage in fat cells. As described above, C-2-labeled citrate, formed from [2-^{13}C]acetate, is cleaved to C-3-labeled oxaloacetate, which may be decarboxylated to yield C-3-labeled pyruvate and lactate.

The commonly accepted idea of the glutamate–glutamine or GABA–glutamine cycle implies glial uptake of extracellular glutamate or GABA with subsequent conversion to glutamine. Glutamine in turn is exported to neurons and converted back to glutamate or GABA (Van den Berg, 1973; Hertz, 1979; Duce and Keen, 1983; Paulsen et al., 1988; Yudkoff et al., 1988; Paulsen and Fonnum, 1989). However, several workers have shown conversion of glutamate or GABA into pyruvate and lactate in vitro (Balázs et al., 1970; Sonnewald et al., 1993*a*; Bachelard et al., 1994). As the present study shows glial conversion of TCA cycle intermediates into lactate, it may be hypothesized that glutamate and GABA, taken up by astrocytes in vivo, to some extent are also converted to lactate. This could

be a security valve lest the glial TCA cycle be flooded with metabolites derived from transmitter amino acids. Such a mechanism could be of special importance after hypoxia, when large amounts of glutamate are shifted from the neuronal to the glial compartment (Aas et al., 1993). Lactate, in turn, may be metabolized by neurons (Izumi et al., 1994; Maran et al., 1994) and converted to glutamate (O'Neal and Koeppe, 1966), completing a glutamate/GABA–lactate cycle, in addition to the glutamate/GABA–glutamine cycle referred to previously. Formation of pyruvate from TCA cycle intermediates was recently inferred from the labeling pattern in glutamate and glutamine obtained with [1,2-^{13}C]acetate (Cerdan et al., 1990). Although acetate is metabolized by glia (see above), the authors concluded that this formation of pyruvate was neuronal because glutamate, but not glutamine, was labeled from the pyruvate thus formed. An alternative explanation may be, however, that the pyruvate was formed from glial TCA cycle intermediates, converted to lactate, and subsequently transferred to neurons and metabolized to glutamate as described above, without being converted to glutamine in glia.

Glial formation of lactate from TCA cycle intermediates has another interesting implication in that lactate has been reported to cause vasodilation in cerebral vessels, even at neutral pH levels (Laptook et al., 1988). As astrocytic processes envelop cerebral vessels, astrocytic conversion of extracellular glutamate into lactate may be one mechanism coupling cerebral blood flow to neuronal activity.

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PAPER V

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Selective Inhibition of the Tricarboxylic Acid Cycle of GABAergic Neurons with 3-Nitropropionic Acid In Vivo

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Abstract: The effects of 3-nitropropionic acid (3-NPA), an inhibitor of succinate dehydrogenase, on cerebral metabolism were investigated in mice by NMR spectroscopy. 3-NPA, 180 mg/kg, caused a dramatic buildup of succinate. Succinate was labeled 5.5 times better from [1-^{13}C]glucose than from [2-^{13}C]acetate, showing a predominantly neuronal accumulation. [1-^{13}C]Glucose labeled GABA in the C-2 position only, compatible with inhibition of the tricarboxylic acid (TCA) cycle associated with GABA formation, at the level of succinate dehydrogenase. Aspartate was not labeled by [1-^{13}C]glucose in 3-NPA–intoxicated animals. In contrast, [1-^{13}C]glucose labeled glutamate in the C-2, C-3, and C-4 positions showing uninhibited cycling of label in the TCA cycle associated with the large, neuronal pool of glutamate. The labeling of glutamine, and hence GABA, from [2-^{13}C]-acetate showed that the TCA cycle of glial cells was unaffected by 3-NPA and that transfer of glutamine from glia to neurons took place during 3-NPA intoxication. The high ^{13}C enrichment of the C-2 position of glutamine from [1-^{13}C]glucose showed that pyruvate carboxylation was active in glia during 3-NPA intoxication. These findings suggest that 3-NPA in the initial phase of intoxication fairly selectively inhibited the TCA cycle of GABAergic neurons; whereas the TCA cycles of glia remained uninhibited as did the TCA cycle associated with the large neuronal pool of glutamate, which includes glutamatergic neurons. This may help explain why the caudoputamen, which is especially rich in GABAergic neurons, selectively undergoes degeneration both in humans and animals intoxicated with 3-NPA. Further, the present results may be of relevance for the study of basal ganglia disorders such as Huntington's disease. **Key Words:** 3-Nitropropionic acid—Tricarboxylic acid cycle—Succinate dehydrogenase—GABA—Glutamate—NMR spectroscopy.
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3-Nitropropionic acid (3-NPA), a plant and fungal toxin, produces basal ganglia degeneration and extrapyramidal symptoms in humans (Ludolph et al., 1991; Liu et al., 1992) as well as in experimental animals (Gould and Gustine, 1982; Gould et al., 1985; Hamilton and Gould, 1987*a*). For this reason 3-NPA intoxication has been suggested as a model for Huntington's disease (Brouillet et al., 1993; Wüllner et al., 1994). 3-NPA is a suicide inhibitor of succinate dehydrogenase (EC 1.3.99.1) (Alston et al., 1977; Coles et al., 1979) and a reversible inhibitor of fumarase (EC 4.2.1.2) (Porter and Bright, 1980). Although the basal ganglia are preferentially destroyed, 3-NPA inhibits succinate dehydrogenase in the whole brain as well as in the liver and heart (Gould and Gustine, 1982; Gould et al., 1985). In a similar manner, congenital dysfunction of the succinate dehydrogenase complex in humans results in selective degeneration of the basal ganglia although the enzyme is dysfunctional throughout the body (Martin et al., 1988). This suggests that basal ganglia are especially susceptible to interference with succinate dehydrogenase activity. Several studies indicate that 3-NPA–induced neurodegeneration involves an excitotoxic mechanism because glutamate antagonists inhibit the toxicity of 3-NPA in cultured neurons (Ludolph et al., 1992; Weller and Paul, 1993), and because acute intoxication with 3-NPA leads to activation of striatal NMDA receptors as reflected by increased binding of [^{3}H]MK-801 (Wüllner et al., 1994). Further, decortication with destruction of the glutamatergic corticostriatal pathways inhibits 3-NPA–induced degeneration of the basal ganglia in vivo (Beal et al., 1993).

The present study was undertaken to investigate the metabolic consequences of acute 3-NPA intoxication in the time period immediately preceding development of morphological changes. Recumbency in mice and rats after 3-NPA administration heralds the onset of morphological changes (Gould and Gustine, 1982; Hamilton and Gould, 1987*a,b*). We therefore administered [1-^{13}C]glucose or [2-^{13}C]acetate to mice injected with 3-NPA in the time period preceding recumbency. The ^{13}C labeling patterns in amino acids and

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Abbreviations used: 3-NPA, 3-nitropropionic acid; TCA cycle, tricarboxylic acid cycle.

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related metabolites were analyzed by NMR spectroscopy. In the brain, glucose is primarily metabolized by neurons, whereas acetate is generally believed to be metabolized by glia (Berl, 1973; Van den Berg, 1973; Minchin and Beart, 1975; Muir et al., 1986; Hassel et al., 1992, 1995*a*; Sonnewald et al., 1993). The labeling pattern obtained with the two ^{13}C-labeled substrates, therefore, illustrates the neuronal and glial metabolism, respectively.

MATERIALS AND METHODS

Materials

Female NMRI mice (Bomholt, Ry, Denmark) were kept in groups of 10 per cage, at 21°C and 40% relative humidity, with a 12-h light/dark cycle and with free access to food and tap water. The animals weighed 30–40 g at the time of the study when they were 4 months old. 3-NPA, [1-^{13}C]glucose, and sodium [2-^{13}C]acetate, the latter two compounds with 99% ^{13}C enrichment, were from Sigma (St. Louis, MO, U.S.A.). Before experiments, 3-NPA was dissolved in physiological saline to a concentration of 20 mg/ml. [1-^{13}C]Glucose and sodium [2-^{13}C]acetate were dissolved in double-distilled water to a concentration of 300 m*M*. pH of all solutions was adjusted to 7.4.

Experimental procedures

Mice were fasted overnight and given a subcutaneous injection of 180 mg/kg 3-NPA. This dose is in excess of the LD_{50} for 3-NPA in mice, 120 mg/kg (Gould and Gustine, 1982), and is comparable with the dose used by Hamilton and Gould (1987*a*,*b*) in rats (30 mg/kg), which is in excess of the LD_{50} in rats, 22 mg/kg (Ludolph et al., 1991). At 10 min the mice were injected in a tail vein with 0.25 ml of a solution of [1-^{13}C]glucose (n = 6) or sodium [2-^{13}C]acetate (n = 6). At 25 min the animals were decapitated. A second group of animals (n = 5), which was injected subcutaneously with 3-NPA, received 0.25 ml [1-^{13}C]glucose at 30 min and was killed at 45 min. Immediately after decapitation the heads were dropped in liquid N_2. The brains were removed in the frozen state and homogenized in 3 ml 7% perchloric acid. Protein was removed by centrifugation at 12,000 *g* for 20 min and was redissolved in 0.1 *M* NaOH with 0.2% sodium dodecyl sulfate (Sigma). The supernatant was neutralized with 9 *M* KOH, and the precipitate, $KClO_4$, was removed by centrifugation (12,000 *g* for 20 min).

Amino acids were determined by HPLC after derivatization with *o*-phthaldialdehyde and with α-aminoadipic acid as internal standard (Paulsen et al., 1987); whereas lactate and glucose were determined by reflectance spectrophotometry using a DT60 Echtachem (Kodak). Protein was quantified by the method of Lowry et al. (1951). For ^{13}C and ^{1}H NMR spectroscopy, the brain extracts were lyophilized to dryness and redissolved in 500 μl D_2O with 0.5 μl dioxane as internal standard. The total level of succinate was quantified by ^{1}H NMR spectroscopy using a Bruker AM-500 spectrometer. ^{1}H NMR spectra (500 MHz) were accumulated with 16K data points, 40° pulse angle, 15-s relaxation delay, and a sweep width of 5 kHz. A line broadening of 0.3 Hz was applied. Sixty-four scans were accumulated per ^{1}H NMR spectrum. Because unidentified peaks interfered with the dioxane peak in the ^{1}H NMR spectra, the lactate peak was used as internal standard, lactate having been quantified spectrophotometrically. Inverse decoupled 125.7 MHz ^{13}C NMR spectra were accumulated on the same instrument using a 30° pulse angle and 31 kHz spectral width with 64K data points. The acquisition time was 1.049 s, and an additional relaxation delay of 10 s was used. Correction for saturation and residual nuclear Overhauser enhancement was not necessary for the resonances investigated. For each spectrum, ~4,000 scans were accumulated. A line broadening of 1–5 Hz was used. All spectra were recorded at ambient temperature.

The percent enrichment of the various carbon positions of the metabolites was determined by subtracting the naturally abundant ^{13}C (1.1% of total carbon) from the total amount of ^{13}C as determined from the ^{13}C NMR spectra and dividing by the total amount of the metabolite (Badar-Goffer et al., 1990). Differences between groups of animals were analyzed statistically with Student's *t* test. Differences in ^{13}C enrichment between carbon positions within the same molecule were analyzed with the paired *t* test. Control values in Tables 2 and 3 are from Hassel et al. (1995*a*). Control animals received the same amount of [1-^{13}C]glucose or sodium [2-^{13}C]acetate as 3-NPA–treated animals and were killed at 15 min.

The experimental procedure adhered strictly to the national and institutional ethics guidelines for animal research.

RESULTS

Behavioral changes

During the first 10 min after subcutaneous injection of 3-NPA, animals tended to lie down more than their uninjected littermates, but they were readily awakened and exhibited a steady gait. At 10 min, animals received an intravenous injection of [1-^{13}C]glucose or sodium [2-^{13}C]acetate. From the 10th to the 20th minute, the tendency to lie down was pronounced, and most of the animals developed an unsteady gait. Thereafter, the animals lay still in the prone position with short episodes of side-to-side movements of the head and paddling movements of the forelimbs. The animals could still be aroused to walk about the cage, but arousal could also elicit paddling. Animals were killed at 25 min. Respiration was unaffected throughout the survival time. The animals in this group, therefore, showed signs of cerebral impairment corresponding to stages I and II of neurological dysfunction induced by 3-NPA (Hamilton and Gould, 1987*a*), meaning that in these animals [1-^{13}C]glucose and [2-^{13}C]acetate were metabolized before morphological alterations had occurred in the brain. A second group of animals was injected intravenously with [1-^{13}C]glucose at 30 min and killed at 45 min after administration of 3-NPA. From the 30th minute after injection of 3-NPA until they were killed these animals developed a deep, rapid respiration and motor activity became progressively depressed; eventually they could not be aroused. This state corresponded to stage III of cerebral impairment induced by 3-NPA (Hamilton and Gould, 1987*a*), which suggests the onset of morphological changes in the brain. The animals also exhibited short episodes of spontaneous paddling, and shortly before decapitation three of five animals had general convulsions. The time

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TABLE 1. *Levels of succinate, amino acids, and related metabolites (nmol/mg of protein) in the brains of mice treated with 3-NPA*

	Succinate	Glutamate	Glutamine	Aspartate	GABA	Alanine	Lactate	Glucose
Control								
[1-^{13}C]Glucose (8)	ND	84.2 ± 3.5	37.6 ± 2.1	23.8 ± 1.2	15.9 ± 0.9	3.8 ± 0.2	30.2 ± 3.0	29.7 ± 1.5
NPA + [1-^{13}C]glucose (6)	18.6 ± 1.2	76.5 ± 4.9	44.6 ± 2.2[b]	26.8 ± 2.7	20.5 ± 1.6[b]	6.2 ± 0.6[c]	40.7 ± 3.8[b]	30.3 ± 1.6
NAP + [1-^{13}C]glucose (5)[a]	36.3 ± 3.4	68.9 ± 2.9[b]	43.2 ± 2.4	18.0 ± 0.8[c]	23.1 ± 1.0[c]	7.7 ± 0.7[c]	54.3 ± 3.4[c]	31.7 ± 1.7
Control								
[2-^{13}C]Acetate (7)	ND	103.1 ± 2.2	43.6 ± 3.0	24.1 ± 1.0	19.8 ± 0.6	4.4 ± 0.2	34.5 ± 1.7	29.3 ± 0.2
NPA + [2-^{13}C]acetate (6)	13.6 ± 1.2	71.6 ± 3.6[e]	36.4 ± 2.2	26.1 ± 0.8	17.1 ± 0.9[d]	3.9 ± 0.2	29.4 ± 3.0	21.6 ± 1.0[e]

Fasted mice were injected subcutaneously with 3-NPA, 180 mg/kg. At 10 min, 0.25 ml 300 m*M* sodium [2-^{13}C]acetate or [1-^{13}C]glucose was injected intravenously. At 25 min, animals were killed. Controls were fasted mice injected with [2-^{13}C]acetate or [1-^{13}C]glucose and killed at 15 min (taken from Hassel et al., 1995*a*). Number of experiments is given in parentheses. Values are means (nmol/mg of protein) ± SEM. ND, not detectable by ^{1}H NMR spectroscopy.

[a] This group was injected intravenously with [1-^{13}C]glucose at 30 min after injection of 3-NPA and killed at 45 min.

Difference from [1-^{13}C]glucose control values at [b] $p < 0.05$ and [c] $p < 0.01$, Student's t test.

Difference from [2-^{13}C]acetate control values at [d] $p < 0.05$ and [e] $p < 0.01$, Student's t test.

course of behavioral changes was quite similar from one animal to another.

Cerebral levels of succinate, amino acids, glucose, and lactate

3-NPA caused a dramatic increase in the cerebral level of succinate (Table 1; Fig. 1). The level of succinate increased further (by 100%) when the survival time was prolonged from 25 to 45 min (Table 1). Malate C-3 was clearly visible in control spectra but could not be seen in spectra from 3-NPA–treated mice (Fig. 1). In the [2-^{13}C]acetate-injected animals 3-NPA caused a reduction in the levels of glutamate, GABA, and glucose (Table 1). In the mice that received [1-^{13}C]glucose at 10 min after injection of 3-NPA, levels of GABA, alanine, glutamine, and lactate were elevated. In mice that received [1-^{13}C]glucose at 30 min after injection of 3-NPA, the levels of aspartate and glutamate were significantly decreased, whereas the levels of GABA, alanine, and lactate were further increased.

^{13}C enrichment of cerebral metabolites

In 3-NPA–treated mice injected with [1-^{13}C]-glucose, ^{13}C enrichment was detected in succinate, glutamate, glutamine, and GABA (Table 2). The enrichment of the C-2 + C-3 in succinate was 21.95 ± 3.00%. Glutamate and glutamine were labeled in the C-2, C-3, and C-4 positions, in a manner qualitatively similar to the labeling pattern in controls, although the percent enrichment of the various carbon positions tended to be lower than in controls (Table 2). The glutamine/glutamate enrichment ratio was 0.37 ± 0.07, as calculated from the enrichment of the C-4 positions. In contrast to glutamate and glutamine, GABA was labeled in the C-2 only (which corresponds to glutamate C-4), and aspartate was not detectably labeled either in the C-2 or in the C-3.

The C-3/C-4 enrichment ratio in glutamate reflects the average number of times that the ^{13}C has passed through the tricarboxylic acid (TCA) cycle (Hassel et al., 1994*a*, 1995*a*). This ratio, as obtained with [1-^{13}C]glucose, was 0.29 ± 0.03, which was not significantly different from controls (Table 3). [1-^{13}C]-Glucose labeled the C-2 of glutamate and glutamine more strongly than the C-3 position. With [1-^{13}C]-glucose as the labeled precursor, the enrichment of glutamine C-2 (minus that of the C-3) relative to the enrichment of glutamine C-4, indicates the flux of pyruvate through pyruvate carboxylase relative to that through pyruvate dehydrogenase in glia (Shank et al., 1993; Hassel et al., 1995*a*). This ratio gave a flux of pyruvate through pyruvate carboxylase of 44 ± 7% (means ± SEM, n = 6) of that through pyruvate dehydrogenase in glia during 3-NPA intoxication, which was very similar to controls, 41 ± 5% (means ± SEM, n = 6).

In mice injected with [1-^{13}C]glucose at 30 min and killed at 45 min after administration of 3-NPA, the ^{13}C enrichment of succinate (C-2 + C-3) was 8.33 ± 0.45%, which was significantly lower than in animals killed at 25 min ($p < 0.01$) in spite of the larger increase in the total level of succinate (Table 2). This shows formation of succinate from unlabeled precursors. The percent enrichment of the amino acids was also lower in this group. However, even at this late time point, there was labeling of the C-2 and C-3 of glutamate and glutamine, but not of GABA C-3 or C-4, nor of aspartate C-2 or C-3.

The ^{13}C enrichment of cerebral glucose was significantly higher in mice injected with [1-^{13}C]glucose at 10 and 30 min after administration of 3-NPA compared with control animals injected with [1-^{13}C]glucose, i.e., 10.2 ± 1.6 (n = 6) and 11.8 ± 2.9 (n = 5) vs. 4.8 ± 0.5% (n = 6) (means ± SEM; $p < 0.05$). This shows reduced metabolism of [1-^{13}C]glucose during 3-NPA intoxication. The ^{13}C enrichment of cerebral lactate C-3 from [1-^{13}C]glucose was also higher in animals injected with 3-NPA than in controls. In ani-

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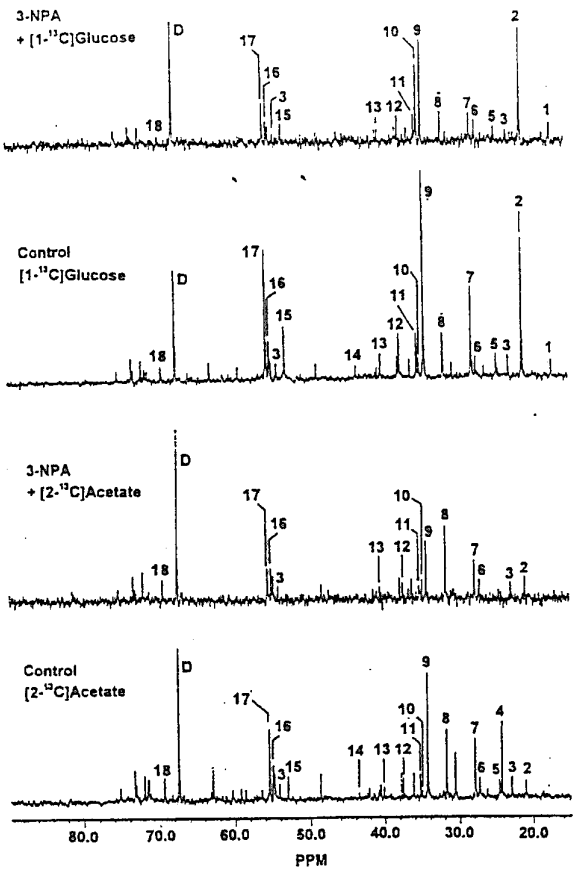

FIG. 1. Representative ^{13}C NMR spectra of extracts of mouse brain after injection of sodium [2-^{13}C]acetate or [1-^{13}C]glucose into mice pretreated with 3-NPA. Mice were injected subcutaneously with 3-NPA 180 mg/kg. At 10 min, 0.25 ml 300 m*M* [1-^{13}C]glucose or sodium [2-^{13}C]acetate was given intravenously. At 25 min, animals were killed. Control mice were injected with [1-^{13}C]glucose or sodium [2-^{13}C]acetate and were killed at 15 min. Peaks identified by numbers: 1, alanine C-3; 2, lactate C-3; 3, *N*-acetylaspartate; 4, acetate C-2; 5, GABA C-3; 6, glutamine C-3; 7, glutamate C-3; 8, glutamine C-4; 9, glutamate C-4; 10, succinate C-2 + C-3; 11, GABA C-2; 12, aspartate C-3; 13, GABA C-4; 14, malate C-3; 15, aspartate C-2; 16, glutamine C-2; 17, glutamate C-2; 18, lactate C-2; D, dioxane (internal standard). The peaks represent both the naturally abundant ^{13}C and the ^{13}C incorporated from [1-^{13}C]glucose and [2-^{13}C]-acetate.

mals injected with [1-^{13}C]glucose at 10 and 30 min after administration of 3-NPA, lactate C-3 was 14.1 ± 1.0 (n = 6) and 14.2 ± 1.1% (n = 5), respectively, compared with a control value of 10.8 ± 1.0% (n = 6; $p < 0.05$; control value from B. Hassel and U. Sonnewald, unpublished data).

Injection of [2-^{13}C]acetate at 10 min after administration of 3-NPA led to ^{13}C enrichment of succinate, glutamate, glutamine, GABA, and aspartate (Table 2). The C-2 + C-3 of succinate had an enrichment of 4.26 ± 0.49%, which was significantly lower than the enrichment obtained with [1-^{13}C]glucose at the same

TABLE 2. *Percent enrichment of various carbon positions in cerebral succinate and amino acids from [2-^{13}C]acetate or [1-^{13}C]glucose after treatment with 3-NPA*

	Succinate	Glutamate			Glutamine		GABA		Aspartate	
	C-2 + C-3	C-2	C-3	C-4	C-2	C-3	C-4	C-2	C-3	C-4	C-2	C-3
[1-^{13}C]Glucose Control (6)	ND	3.44 ± 0.41[c]	2.29 ± 0.24	5.79 ± 0.48	1.85 ± 0.25[c]	0.94 ± 0.17	2.62 ± 0.37	6.54 ± 0.67	2.72 ± 0.54	3.19 ± 0.27	4.88 ± 0.31	4.89 ± 0.45
[1-^{13}C]Glucose 3-NPA (6)	21.95 ± 3.00	2.87 ± 0.49[b]	1.77 ± 0.19	5.65 ± 0.31	1.36 ± 0.33[b]	0.94 ± 0.30	2.18 ± 0.59	8.09 ± 1.26	ND	ND	ND	ND
[1-^{13}C]Glucose[a] 3-NPA (5)	8.33 ± 0.45	1.20 ± 0.17	1.00 ± 0.15	3.98 ± 0.47	1.06 ± 0.46	0.30 ± 0.15	1.69 ± 0.38	5.51 ± 0.55	ND	ND	ND	ND
[2-^{13}C]Acetate Control (6)	ND	0.97 ± 0.17[c]	0.82 ± 0.15	2.78 ± 0.29	0.96 ± 0.29	0.97 ± 0.20	5.54 ± 0.44	3.85 ± 0.77	1.68 ± 0.55	1.02 ± 0.30	1.14 ± 0.27	2.00 ± 0.42
[2-^{13}C]Acetate 3-NPA (6)	4.26 ± 0.49	0.76 ± 0.02	0.42 ± 0.16	1.46 ± 0.20	1.14 ± 0.14[b]	0.59 ± 0.09	5.03 ± 1.05	1.46 ± 0.36	ND	ND	0.98 ± 0.49	1.23 ± 0.28

Fasted mice were injected subcutaneously with 3-NPA, 180 mg/kg. At 10 min, 0.25 ml 0.3 *M* [1-^{13}C]glucose or sodium [2-^{13}C]acetate was injected intravenously. Animals were killed at 25 min. ND, not detectable. Control values are from Hassel et al. (1995*a*). Control animals were injected intravenously with the same amount of [1-^{13}C]glucose or sodium [2-^{13}C]acetate as 3-NPA–treated animals. Survival time was 15 min.

[a] This group was injected intravenously with [1-^{13}C]glucose at 30 min after injection of 3-NPA and killed at 45 min.

Difference from the C-3 position in the same molecule at [b] $p < 0.05$ and [c] $p < 0.01$, paired *t* test.

TABLE 3. *The C-3/C-4 labeling ratios in glutamate and glutamine, and the C-3/C-2 labeling ratio in GABA, obtained with [1-^{13}C]glucose or [2-^{13}C]acetate during acute 3-NPA intoxication*

	C-3/C-4 labeling ratio		C-3/C-2 labeling ratio
	Glutamate	Glutamine	GABA
[1-^{13}C]Glucose (6) control	0.38 ± 0.02		0.41 ± 0.11
[1-^{13}C]Glucose (6) 3-NPA	0.29 ± 0.03		0
[2-^{13}C]Acetate (6) control	0.27 ± 0.05	0.13 ± 0.02	0.40 ± 0.01
[2-^{13}C]Acetate (6) 3-NPA	0.30 ± 0.12	0.12 ± 0.02	0

Fasted mice were injected subcutaneously with 3-NPA 180 mg/kg. At 10 min, 0.25 ml 300 m*M* sodium [2-^{13}C]acetate or [1-^{13}C]glucose was injected intravenously. At 25 min, animals were killed. Values represent ratios of ^{13}C enrichment between the C-3 and the C-4 positions in glutamate and glutamine, and between the corresponding C-3 and C-2 in GABA, means ± SEM. In parentheses are the numbers of animals. Control values are from Hassel et al. (1995*a*). The C-3/C-4 labeling ratio in glutamate and glutamine (or C-3/C-2 in GABA) reflects the degree of cycling of label in a TCA cycle (see text).

time point ($p < 0.001$). As seen with [1-^{13}C]glucose, [2-^{13}C]acetate labeled glutamate and glutamine in the C-2, C-3, and C-4 positions, whereas GABA was labeled in the C-2 position only. The glutamine/glutamate enrichment ratio was 3.85 ± 0.61 as calculated from the enrichment of the C-4 positions. The C-3/C-4 enrichment ratios in glutamine and glutamate were 0.12 ± 0.02 and 0.30 ± 0.12, respectively, with [2-^{13}C]acetate. These values, which reflect the degree of cycling of label in a TCA cycle (Hassel et al., 1994*a*), were very similar to controls (Table 3). [2-^{13}C]-Acetate yielded a higher enrichment of glutamine C-2 than of the C-3. In contrast to [1-^{13}C]glucose, [2-^{13}C]acetate labeled aspartate. The percent enrichments of the C-2 and C-3 positions in aspartate were not significantly different, in agreement with scrambling of label between the C-2 and C-3 positions in the symmetrical succinate step of the TCA cycle.

DISCUSSION

This study describes some acute metabolic effects of intoxication with 3-NPA. It is most remarkable that succinate accumulated, which is in agreement with inhibition of succinate dehydrogenase by 3-NPA. The glutamine/glutamate enrichment ratio became less than one with [1-^{13}C]glucose and greater than one with [2-^{13}C]acetate, confirming the supposition that [1-^{13}C]glucose was primarily metabolized by neurons, whereas [2-^{13}C]acetate was metabolized by the small compartment that corresponds to glia (Balázs et al., 1970; Berl, 1973; Van den Berg, 1973; Minchin and Beart, 1975; Muir et al., 1986; Hassel et al., 1992, 1995*a*; Sonnewald et al., 1993). Succinate was labeled 5.5 times better from [1-^{13}C]glucose than from [2-^{13}C]acetate. The much higher enrichment of succinate from [1-^{13}C]glucose than from [2-^{13}C]acetate, therefore, indicates that succinate primarily built up in neurons during 3-NPA intoxication. In 3-NPA–intoxicated animals, GABA was labeled in the C-2 position only. Such labeling does not require the operation of the TCA cycle beyond the succinate step; whereas GABA C-3 and C-4 are labeled only when the ^{13}C has completed full turns through the TCA cycle (Badar-Goffer et al., 1990; Shank et al., 1993). Labeling of GABA C-2 only is therefore compatible with inhibition of the TCA cycle of GABAergic neurons at the level of succinate dehydrogenase.

In contrast, [1-^{13}C]glucose labeled glutamate both in the C-2, C-3, and C-4 positions, with a C-3/C-4 enrichment ratio similar to control. Glutamate C-2 and C-3 are labeled after cycling of label in the TCA cycle, which shows that the TCA cycle associated with the large neuronal pool of glutamate, which includes glutamatergic neurons (Ottersen and Storm-Mathisen, 1985), was virtually uninhibited by 3-NPA. It should be noted that the pool of glutamate in GABAergic neurons is quite small (Balázs et al., 1970; Cremer et al., 1978; Ottersen and Storm-Mathisen, 1985; Ottersen, 1989) and that this glutamate, which is the precursor for GABA, could not have belonged to the pool of glutamate that was labeled in the C-2 and C-3, because GABA itself was not labeled in the corresponding C-4 and C-3 positions.

3-NPA completely prevented labeling of aspartate from [1-^{13}C]glucose in agreement with inhibition of succinate dehydrogenase. This is especially interesting because aspartate is concentrated in some populations of GABAergic neurons, whereas it tends to be low in glutamatergic neurons (Ottersen and Storm-Mathisen, 1985; Hassel et al., 1992, 1995*b*). It may, therefore, be that the lack of labeling of the C-3 and C-4 of GABA and of the C-2 and C-3 of aspartate is related to inhibition of the same TCA cycle, i.e., that of GABAergic neurons. It is interesting that Cremer (1964) found that another inhibitor of succinate dehydroge-

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nase, malonate, abolished labeling of aspartate and gave a greater reduction in the labeling of GABA than of glutamate in cortical slices incubated with [U-^{14}C]-glucose.

The cerebral metabolism of acetate takes place in glia (Berl, 1973; Muir et al., 1986; Hassel et al., 1992; Sonnewald et al., 1993), as does the formation of glutamine (Martinez-Hernandez et al., 1977). Labeling of glutamine C-2 and C-3 from [2-^{13}C]acetate, therefore, requires cycling of label in the glial TCA cycle. That [2-^{13}C]acetate gave a C-3/C-4 enrichment ratio in glutamine that was similar to control shows that the glial TCA cycle remained uninhibited by 3-NPA. The labeling of GABA from [2-^{13}C]acetate, which is thought to occur via transfer of labeled glutamine from glia to neurons (Van den Berg and Garfinkel, 1971; Sonnewald et al., 1993), shows that export of glutamine from glia to GABAergic neurons took place during 3-NPA intoxication. Complementary to the export of glutamine is the anaplerotic activity of the mitochondrial enzyme pyruvate carboxylase (Waelsch et al., 1964; Hassel et al., 1994*a*), which has a glial localization (Yu et al., 1983). This enzyme was operative at control levels during 3-NPA treatment. These results agree with the finding by Hamilton and Gould (1987*a*) that glial mitochondria are morphologically well preserved in acute intoxication with 3-NPA when neuronal mitochondria may be severely deranged. The labeling of succinate from [2-^{13}C]acetate during 3-NPA intoxication probably reflects transfer of labeled glutamine to GABAergic neurons with subsequent conversion to succinate. The labeling of aspartate from [2-^{13}C]acetate, on the other hand, may reflect formation of aspartate in glia.

Inhibition of the TCA cycle by 3-NPA may have affected cerebral levels of ATP (Erecińska and Nelson, 1994). ATP was not measured, however, because some degree of hypoxia inevitably occurred from the time of decapitation until the brain became frozen (Pontén et al., 1973), which would have been a significant source of error. Further, from the foregoing it should be clear that different cell types are differently affected by 3-NPA, and this would not be reflected in the overall energy state of the brain. Some of the metabolic activities that have been described are, however, ATP dependent or energy yielding and give clues as to the energy state of different cell types. The glial enzymes glutamine synthetase and pyruvate carboxylase are ATP dependent (Schomburg and Salzmann, 1990), and clear evidence of their function indicates strongly that glia was largely unaffected by 3-NPA in terms of energy reserves. Glutamate was labeled in the C-2 and C-3 from [1-^{13}C]glucose, which shows the operation of the TCA cycle in cells associated with the large neuronal pool of glutamate, i.e., glutamatergic neurons (Ottersen and Storm-Mathisen, 1985). These neurons were therefore capable of ATP generation. The TCA cycle of GABAergic neurons, on the other hand, was clearly inhibited by 3-NPA, as shown by the lack of labeling of the GABA C-3 and C-4, and these cells would be expected to be energy deficient.

Inhibition of the TCA cycle of GABAergic neurons may explain some of the behavioral changes seen in 3-NPA intoxication. Energy failure of GABAergic neurons will lead to depolarization (Riepe et al., 1992), which probably entails excessive release of GABA. Release of GABA from cortical slices incubated with malonate, another inhibitor of succinate dehydrogenase, has been reported previously (Cremer, 1964). An increase in GABAergic neurotransmission could underlie the depression of motor activity and the somnolence of the initial phase of 3-NPA intoxication (Gould and Gustine, 1982; Hamilton and Gould, 1987*a*). Interference with normal GABAergic neurotransmission may, however, increase excitability and lower the threshold for paroxysmal activity and convulsions, as seen in the animals with the longer survival time after injection of 3-NPA.

Inhibition of the TCA cycle probably also leads to increased susceptibility to excitotoxic injury in GABAergic neurons. The depolarization induced by energy failure leads to relief of the magnesium block of the NMDA receptor (Novelli et al., 1988) and its subsequent activation, as demonstrated recently in rats acutely intoxicated with 3-NPA (Wüllner et al., 1994). The caudoputamen is rich in GABAergic neurons. Moreover, these neurons receive a strong glutamatergic input via corticostriatal projections (Divac et al., 1977; McGeer et al., 1977). The inhibition of energy production in GABAergic neurons, together with a glutamatergic stimulation that could be augmented due to increased excitability, may explain why the caudoputamen is especially prone to neurodegeneration after intoxication with 3-NPA (Gould and Gustine, 1982; Hamilton and Gould, 1987*a*; Ludolph et al., 1991; Brouillet et al., 1993). An additional neurotoxic mechanism may be the opening of the blood–brain barrier with efflux of plasma proteins, especially albumin, which is seen in the caudoputamen in 3-NPA intoxication (Hamilton and Gould 1987*b*), and which may itself be a consequence of glutamate overstimulation (Dietrich et al., 1992; Nag, 1992). Albumin strongly potentiates the excitotoxic effect of glutamate in cultured neurons (Eimerl and Schramm, 1991), and intrastriatal injection of albumin causes a dose-dependent neurodegeneration in vivo (Hassel et al., 1994*b*).

Huntington's disease is characterized by loss of neurons in several brain areas, most notably in the caudoputamen where GABAergic cells become depleted (Graveland et al., 1985), a process that may be mediated by excessive NMDA-receptor activation (Young et al., 1988). 3-NPA intoxication has been suggested as a model for Huntington's disease (Brouillet et al., 1993; Wüllner et al., 1994). The present study, which shows a preferential effect of 3-NPA on the TCA cycle activity of GABAergic neurons, supports the idea of 3-NPA intoxication as a model for Huntington's disease.

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PAPER VI

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Metabolic Differences Between Primary Cultures of Astrocytes and Neurons from Cerebellum and Cerebral Cortex. Effects of Fluorocitrate

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Astrocytes and neurons cultured from mouse cerebellum and cerebral cortex were analyzed with respect to content and synthesis of amino acids as well as export of metabolites to the culture medium and the response to fluorocitrate, an inhibitor of aconitase. The intracellular levels of amino acids were similar in the two astrocytic populations. The release of citrate, lactate and glutamine, however, was markedly higher from cerebellar than from cortical astrocytes. Neurons contained higher levels of glutamate, aspartate and GABA than astrocytic cultures. Cortical neurons were especially high in GABA and aspartate, and the level of aspartate increased specifically when the extracellular level of glutamine was elevated. Fluorocitrate inhibited the TCA cycle in the astrocytes, but was less effective in cerebellar neurons. Whereas neurons responded to fluorocitrate with an increase in the formation of lactate, reflecting glycolysis, astrocytes decreased the formation of lactate in the presence of fluorocitrate, indicating that astrocytes to a high degree synthesize pyruvate and hence lactate from TCA cycle intermediates.

KEY WORDS: Neurons; astrocytes; amino acids; citrate; fluorocitrate.

INTRODUCTION

The study of cerebral amino acid metabolism in the intact brain is complicated by the variety of cells (neurons, glia, cells of the vasculature) that constitute the brain tissue. Further, brain cells show great metabolic heterogeneity according to the type of neurotransmission they are involved in. Cultured brain cells can give valuable information on the relative contribution of different cell types to the overall metabolic picture (1–5). The aim of the present study was to provide information on the content and synthesis of amino acids and related metabolites in different populations of astrocytes and neurons. Cultured cerebellar neurons are predominantly glutamatergic, whereas cultured cortical neurons are mainly GABAergic (2). Therefore the content of amino acids and the incorporation of radiolabel from [1-^{14}C]glucose into amino acids were determined in primary cultures of astrocytes and neurons from the cerebellum and the cerebral cortex of the mouse. Release of citrate from cultured astrocytes but not from neurons was demonstrated by Sonnewald et al. (6). We therefore wished to see whether regional differences existed in the astrocytic release of citrate and other metabolites. To further unveil possible functional differences between astrocytes from the two brain regions, these cells were subjected to the metabolic stress of fluorocitrate, an inhibitor of aconitase (EC 4.2.1.3) of the tricarboxylic acid (TCA) cycle (7,8).

Within a narrow dose range, fluorocitrate has been shown to be a selective inhibitor of glial cell metabolism

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in vivo (9,10). In order to study this specificity, the effects of fluorocitrate on neurons and astrocytes were compared.

EXPERIMENTAL PROCEDURE

Materials. Pregnant (15th gestational day), new born and 7-day-old mice were obtained from the animal quarters of the Panum Institute, University of Copenhagen. Plastic tissue culture dishes were from NUNC A/S (Denmark). Nitex nylon sieves were from Schweiz Seidengasefabrik AG, Thal (Switzerland). Fetal calf serum was from Seralab Ltd. (Sussex, UK). Poly-L-lysine (mol. wt. > 300,000), trypsin, trypsin inhibitor, DNase, fluorocitrate and amino acids were from Sigma Chemicals Corp. (St. Louis, MO), insulin from NOVO-Nordisk (Denmark), and penicillin from Leo (Denmark). [1-^{14}C]D-glucose (specific activity 7.5 mCi/mmol) was from Dupont-NEN (Germany), and enzymes and coenzymes from Boehringer Mannheim (Germany). All other chemical were of the purest grade available from regular commercial sources. Fluorocitrate was prepared from the barium salt as described previously (9).

Cell Cultures. Astrocytes were cultured essentially as described by Hertz et al. (11). Prefrontal cerebral cortex was taken from new born mice (cortical astrocytes) and cerebellum from 7-day-old mice (cerebellar astrocytes). The tissue samples were passed through sterile 80 μm pore size Nitex nylon sieves into a slightly modified Dulbecco's medium containing 20% (v/v) fetal calf serum and 2.5 mM glutamine, and were plated in Petri dishes coated with poly-L-lysine. The astrocytes were grown for a total of 3 weeks. The medium was changed two days after inoculation and subsequently twice a week. The last 24 hours the astrocytes were incubated in a culture medium which contained either no or 2.5 mM glutamine to see how extracellular glutamine might influence the intracellular levels of amino acids in astrocytes.

Cerebellar neurons and cortical neurons were cultured from cerebella of 7-day-old mice and from prefrontal cortices from 15-day-old mice embryos, respectively (12,13). After mild trypsinization followed by trituration in a DNase solution containing a trypsin inhibitor from soybeans, cells were suspended in neuronal culture medium containing 10% (v/v) fetal calf serum and 0.2 or 0.8 mM glutamine. The two concentrations of glutamine were chosen to evaluate the effect of extracellular glutamine on the levels of intracellular amino acids. A glutamine concentration as high as 2.5 mM (which was used in the astrocytes) would have caused excessive neuronal degeneration. After 48 hours in culture 20 μM cytosine arabinoside was added to the culture medium to prevent astrocytic proliferation (14,15). Cells were cultured for 7 days at which time the neurons are functionally differentiated (16). Under these culture conditions the cerebellar cultures consist mainly (>85%) of granule cells and 5–10% GABAergic neurons (17). The cerebral cortical cultures consist mainly (>80%) of GABAergic neurons (16).

Incubation with Fluorocitrate. To study the metabolic effect of fluorocitrate on cultured brain cells, astrocytes were incubated in fresh astrocytic medium (6 mM glucose, no glutamine) with fluorocitrate in the following concentrations: 0, 5, 20, and 100 μM. After 24 hours the media were collected for determination of glucose, lactate, citrate, ammonia and amino acids.

The cells were then incubated in 1 ml fresh medium containing 0.25 mM glucose and 2 μCi [1-^{14}C]glucose for 15 minutes. In the case of the cerebellar neuronal cultures, fluorocitrate, in the concentrations given above, was added directly to the culture medium. This medium originally contained 0.8 mM glutamine. After 24 hours the media were collected and replaced with fresh medium containing [1-^{14}C]glucose for 15 minutes. Five cultures, neuronal or astrocytic, were used per concentration of fluorocitrate. At the end of the incubation period the cells were rapidly washed with ice-cold, phosphate-buffered saline. 500 μl ice-cold 70% ethanol was then added, and the cells were collected with a teflon scraper. Protein was removed by centrifugation. The supernatants, which contained the intracellular amino acids, were lyophilized to dryness, redissolved in 60 μl double distilled water, and frozen at −20°C until analysis.

Biochemical Analysis. Amino acids were analyzed in the cell extracts and culture media by HPLC after derivatization with *o*-phthaldialdehyde, using α-amino adipic acid as internal standard (9). Standard solutions of the amino acids under study were run frequently as external standards. To determine the incorporation of ^{14}C from [1-^{14}C]glucose into the various amino acids, the amino acids were separated by HPLC over 60 minutes after derivatization with *o*-phthaldialdehyde (10). The eluent from the HPLC column was collected in 1-minute fractions containing the separated amino acids, and the radioactivity in the fractions was measured by scintillation counting. This allowed the determination of the base line radioactivity which was subtracted from the activity of the amino acid peaks. Underivatized amino acids and other metabolites were eluted during the first 5 minutes of chromatography, before the elution of the derivatized amino acids. The amounts of glucose, lactate, citrate, and ammonia in the culture media were measured spectrophotometrically with the use of enzymatic kits obtained from Boehringer Mannheim. Protein was dissolved in 0.1 M NaOH with sodium dodecyl sulfate, 2 g/l, and was measured by the method of Lowry et al. (18) using bovine serum albumin as a standard. Data were analyzed for statistical differences by the Student's t-test.

RESULTS

Content of Amino Acids in Different Populations of Astrocytes and Neurons. Release of Amino Acids, Lactate, and Citrate from Cortical and Cerebellar Astrocytes to the Culture Medium. The intracellular levels of amino acids were quite similar in the two types of astrocytes (Table I). Compared to neurons, the astrocytes contained half as much glutamate and two to three times as much glutathione and taurine. GABA was not detected in the astrocytic cultures, which shows that these cultures were free of contamination with GABAergic neurons. When glutamine was present in the culture medium for the last 24 hours, the intracellular level of glutamine was 58 ± 4 and 47 ± 3 nmol/mg protein in cerebellar and cortical astrocytes, respectively. When glutamine was absent, the intracellular level of glutamine was to 16–18 nmol/mg protein (Table I). In the presence of glutamine the level of alanine was 17 ± 2 nmol/mg protein in both astrocytic cultures, in the absence of glutamine the level of alanine was 5–6 nmol/mg protein (Table I). The levels of glutamate and aspartate were unaffected by the absence of glutamine in the astrocytic culture medium (data not shown).

Table I. Content of Amino Acids (nmol/mg Protein) in Extracts of Neuronal and Astrocytic Cultures

	Cerebellar astrocytes 0 mM Gln	Cortical astrocytes 0 mM Gln	Cerebellar neurons 0.2 mM Gln	Cortical neurons 0.2 mM Gln
Glutamate	39.8 ± 4.9†	40.3 ± 4.5**	65.4 ± 7.2**	96.5 ± 4.5
GABA	n.d.	n.d.	19.3 ± 2.4**	75.8 ± 5.8
Aspartate	14.5 ± 1.5	22.0 ± 3.6**	22.2 ± 3.4**	51.2 ± 2.1
Glutamine	15.8 ± 1.2††	18.0 ± 2.6**	4.5 ± 0.3**	1.5 ± 0.1
Alanine	5.1 ± 0.1†	5.9 ± 1.0**	10.4 ± 1.5	11.3 ± 0.6
Taurine	328.2 ± 66.7†	367.0 ± 34.8**	110.1 ± 7.3	145.7 ± 4.3
GSH	35.4 ± 2.4††	32.5 ± 0.6**	8.2 ± 0.6	17.9 ± 1.1

Neurons from mouse cerebellum and cerebral cortex were grown in a medium containing 0.2 mM glutamine (Gln), whereas astrocytes from the two brain regions were grown in medium without glutamine supplementation for the last 24 hours. Values are given as nmol/mg protein, means ± SEM of five measurements. GSH: glutathione, both reduced and oxidized. n.d.: not detected. *indicates difference from corresponding values in cortical neurons, †indicates difference from cerebellar neurons. * and †: $p < 0.05$; ** and ††: $p < 0.01$; *t*-test.

Table II. Amounts of Various Metabolites (nmol/mg Protein) in the Culture Medium of Astrocytes and Neurons Incubated with Fluorocitrate for 24 Hrs

a: Cortical Astrocytes				
µM fluorocitrate	0	5	20	100
Glucose§	13200 ± 300	12200 ± 300*	11600 ± 300**	10600 ± 300**
Lactate	13600 ± 500	11700 ± 200*	10900 ± 700*	9100 ± 800**
Citrate	380 ± 50	700 ± 30**	720 ± 60**	1010 ± 30**
NH_3	440 ± 60	680 ± 110	1220 ± 150**	1630 ± 60**
Glutamine	1730 ± 40	1270 ± 110**	720 ± 60**	80 ± 16**
Alanine	610 ± 10	540 ± 45	390 ± 10**	200 ± 25**
b: Cerebellar Astrocytes				
µM fluorocitrate	0	5	20	100
Glucose§	15800 ± 900†	16400 ± 300	14800 ± 400	14200 ± 400
Lactate	22200 ± 1000††	20400 ± 1100	18600 ± 700*	16300 ± 800**
Citrate	2130 ± 150††	2620 ± 120*	2500 ± 220	3010 ± 220**
NH_3	470 ± 120	1020 ± 110*	1390 ± 75**	1630 ± 90**
Glutamine	2542 ± 153††	2300 ± 110	1410 ± 180**	440 ± 70**
Alanine	980 ± 90††	930 ± 50	920 ± 30	680 ± 60*
c: Cerebellar Neurons				
µM fluorocitrate	0	5	20	100
Glucose§	53100 ± 2080	56100 ± 2460	54200 ± 2770	49300 ± 3150
Lactate	101000 ± 9230	105800 ± 2300	96800 ± 2300	140700 ± 10400*
Citrate	640 ± 45	830 ± 100	910 ± 60**	1220 ± 100**
NH_3	1120 ± 30	1140 ± 40	980 ± 50*	1030 ± 50
Glutamine	2610 ± 180	n.d.	n.d.	2220 ± 80
Alanine	2030 ± 100	2120 ± 50	2230 ± 70	2490 ± 60**

Astrocytes from cerebral cortex and cerebellum as well as cerebellar neurons were incubated with fluorocitrate for 24 hours, whereafter the culture media were analyzed for metabolites. The astrocytic culture medium was not supplemented with glutamine during the incubation with fluorocitrate. Values are given as nmol/mg protein, means ± SEM; n = 5. §: the values for glucose indicate the amount of glucose consumed in the incubation period; n.d.: not determined. *: difference from control; †: difference from cortical astrocytes: * and †: $p < 0.05$; ** and ††: $p < 0.01$.

Some marked differences were seen in the export of metabolites from the two populations of astrocytes to the culture medium. Cerebellar astrocytes released five times more citrate than the cortical astrocytes (Table II a and b). The export of lactate, glutamine and alanine was also higher from the cerebellar astrocytes. The ratio

of lactate formation to glucose consumption was higher in the cerebellar than in the cortical astrocytes (Table II a and b). The cerebellar astrocytes consumed 15,800 nmol of glucose/mg protein. Of these, 11,000 nmol could be accounted for by the 22,000 nmol of lactate formed. Another 4,600 nmol could be accounted for by the formation of 2,100 nmol citrate and 2,500 nmol glutamine. The export of alanine (980 nmol) made up for the rest of the consumed glucose. In cortical astrocytes the formation of lactate, citrate, alanine and glutamine could account for only 70 per cent of the consumption of glucose, which suggests that formation of CO_2 was more prominent in cortical than in cerebellar astrocytes. In these experiments glutamine was not present in the culture medium. The metabolism of other amino acids in the culture medium was not determined, but it was probably not prominent due to the low concentration of amino acids in the culture medium compared to the concentration of glucose.

The cortical neurons contained twice as much aspartate and three times as much GABA as the cerebellar neurons, whereas the level of glutamate in the cortical neurons was only 33% higher than that in the cerebellar neurons (Table I). Cortical and cerebellar neuronal cultures were, however, quite similar with respect to incorporation of radiolabel from [^{14}C]glucose into the amino acids (Table IIIc, data for cortical neurons not shown). Increasing the concentration of glutamine in the neuronal culture medium from 0.2 to 0.8 mM, caused a three to fourfold increase in the intracellular content of glutamine and aspartate in the cortical neurons (to 8.7 $\pm$ 1.3 and 150 $\pm$ 15.1 nmol/mg protein, respectively), whereas the level of glutamate and alanine remained unaltered (results not shown). In the cerebellar neurons such an increase in extracellular glutamine only caused a threefold increase in intracellular glutamine (to 16.3 $\pm$ 1.6 nmol/mg protein) and an 80% increase in alanine (to 17.2 $\pm$ 2.0 nmol/mg protein); the other amino acids did not change significantly. Thus with an increased extracellular concentration of glutamine the level of aspartate in cortical neurons became twice as high as the level of glutamate in these cells, and it became five times higher than the level of aspartate in cerebellar neurons.

The neuronal cultures had been incubated with the same culture medium for one week, so the amounts of metabolites represented the metabolism of that period. These values will only be discussed in terms of the changes in metabolism caused by fluorocitrate.

The effect of Fluorocitrate on Cerebellar and Cortical Astrocytes. In cortical astrocytes fluorocitrate caused a dose-dependent increase in the export of citrate whereas the release of glutamine was reduced in accordance with an inhibition of aconitase (7). The formation of citrate increased by 170%, and the net formation of glutamine was reduced by 97% in the presence of 100 µM fluorocitrate (Table IIa). NH_3 increased dose-dependently in the culture medium, reflecting the reduced capacity for NH_3 fixation during inhibition of glutamine synthesis. Fluorocitrate inhibited in a dose-dependent manner both the metabolism of glucose and the export of lactate and alanine in cortical astrocytes (Table IIa).

In the cerebellar astrocytes fluorocitrate did not significantly affect the consumption of glucose, but even so there was a significant and dose-dependent reduction in the export of lactate and alanine (Table IIb). The formation of citrate increased by 40% in the presence of 100 µM fluorocitrate, whereas the export of glutamine was reduced by 83%. The increase in NH_3 was quite similar to that seen in the cortical astrocytes (Table II a and b).

Fluorocitrate caused a dose-dependent reduction both in the intracellular levels of glutamate and glutamine as well as in their radiolabeling from [1-^{14}C]glucose in both types of astrocytic cultures. With 20 µM fluorocitrate the intracellular levels of glutamate and glutamine was reduced by approximately 40% (Fig. 1 a,b) whereas the radiolabeling of these amino acids was reduced by 60–70% (Table IIIa,b). The radiolabeling of aspartate, however, was not affected by 20 µM fluorocitrate in either type of astrocytic culture (Table IIIa,b). In the presence of 100 µM fluorocitrate the intracellular level of aspartate in cerebellar astrocytes was reduced by only 20% (Fig. 1b) in spite of the 50% reduction in its radiolabeling. Likewise, the radiolabeling of alanine was reduced by 60–75% in the presence of 100 µM fluorocitrate in both astrocytic cultures, although the intracellular levels remained constant. This either shows formation of aspartate and alanine from unlabeled sources in the presence of fluorocitrate, or that the pools of aspartate and alanine had low turnover rates. The intracellular level of serine, which was present in the culture medium, was not altered by fluorocitrate in any of the astrocytic cultures (data not shown).

The Effect of Fluorocitrate on Cerebellar Neurons. In the cerebellar neurons fluorocitrate did not affect the overall consumption of glucose. The export of lactate and alanine, however, increased (Table IIc), and the formation of glutamate from [1-^{14}C]glucose decreased (Table IIIc), indicating a shift from oxidative metabolism to glycolysis. The formation of lactate seen in the presence of 100 µM fluorocitrate was greater than what could be accounted for by the amount of glucose consumed. This shows formation of lactate from other substrates than glucose. These changes contrast with those seen in the

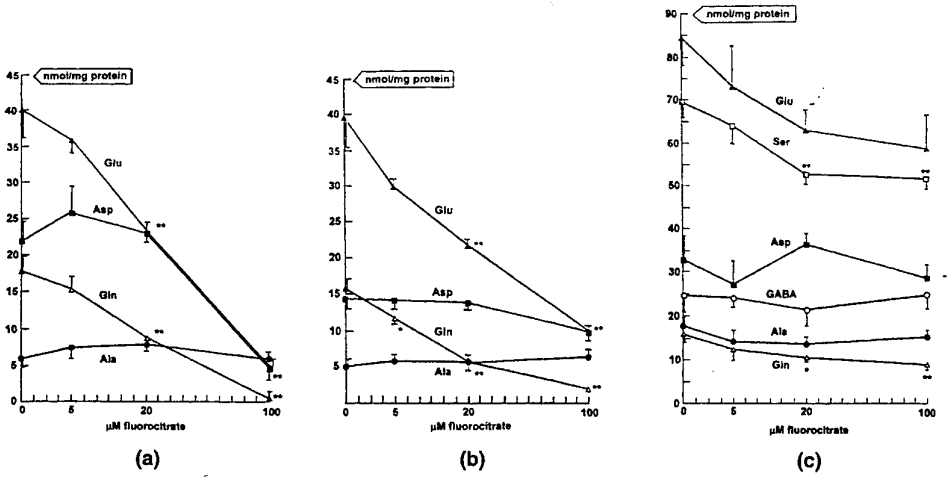

Fig. 1. (a and b): Intracellular levels of amino acids in cultured astrocytes after 24 hours of incubation with 0, 5, 20, or 100 μM fluorocitrate without addition of glutamine. Values are given as nmol/mg protein; means of five culture dishes per concentration of fluorocitrate; vertical bars indicate SEM. *: difference from control; * $p<0.05$; **: $p<0.01$; *t*-test. a: astrocytes from cerebral cortex; b: astrocytes from cerebellum. (c): Intracellular levels of amino acids in cerebellar neurons after 24 hours of incubation with 0, 5, 20, or 100 μM fluorocitrate and with 0.8 mM glutamine (as opposed to the 0.2 mM in Table I) in the incubation medium. Values are given as nmol/mg protein; means of five culture dishes per concentration of fluorocitrate; vertical bars indicate SEM. *: difference from control; * $p<0.05$; **: $p<0.01$; *t*-test.

Table III. Incorporation of Radiolabel into Amino Acids (dpm/mg Protein) in Astrocytic and Neuronal Cultures Exposed to Fluorocitrate

a: Cortical Astrocytes				
μM fluorocitrate	0	5	20	100
Glutamate	18500 ± 730	13600 ± 1400*	7200 ± 900**	2020 ± 160*
Glutamine	3500 ± 700	1680 ± 230*	1000 ± 150*	330 ± 60**
Aspartate	5550 ± 480	4860 ± 530	5560 ± 1600	850 ± 140**
Alanine	1600 ± 240	1600 ± 240	1070 ± 270	460 ± 45**
b: Cerebellar Astrocytes				
μM fluorocitrate	0	5	20	100
Glutamate	24900 ± 1180††	18390 ± 2130*	10200 ± 1360**	2780 ± 220**
Glutamine	4540 ± 290	3080 ± 510*	1190 ± 240**	460 ± 110**
Aspartate	4890 ± 110	4820 ± 620	4160 ± 560	2280 ± 400**
Alanine	2120 ± 120††	1510 ± 270	1000 ± 90**	840 ± 110**
c: Cerebellar Neurons				
μM fluorocitrate	0	5	20	100
Glutamate	44300 ± 2660	46200 ± 2370	25900 ± 740**	20600 ± 790**
Glutamine	960 ± 120	690 ± 60	270 ± 30**	420 ± 60**
Aspartate	8470 ± 1360	9450 ± 1140	6670 ± 870	5550 ± 250
Alanine	1150 ± 150	1410 ± 240	790 ± 130	470 ± 60**
GABA	1580 ± 300	1230 ± 290	70 ± 20**	60 ± 20**

Astrocytes from cerebral cortex and cerebellum as well as cerebellar neurons were incubated with fluorocitrate for 24 hours, whereafter the culture medium was replaced with fresh medium containing 2 μCi [1-^{14}C]glucose for 15 minutes. Values are given as dpm/mg protein, means ± SEM; n = 5. *:$p<0.01$. ††difference from corresponding values in cortical astrocytes, $p<0.01$.

astrocytic cultures, where fluorocitrate caused a decrease in the export of lactate and alanine. In the cerebellar neurons fluorocitrate caused an increase in the formation of citrate, reflecting an inhibition of the TCA cycle. There was, however, no accompanying increase in NH_3 (Table IIc). The radiolabeling of glutamine in cerebellar

granule cells, which was only 2% of the labelling of glutamate, was reduced by 56% (Table IIIc).

With respect to intracellular amino acids fluorocitrate only caused significant decreases in the levels of serine and glutamine (Fig 1c). The level of GABA remained unaltered even in the presence of 100 μM fluorocitrate, although the radiolabeling from [1-^{14}C]glucose was reduced by 96% by 100 μM fluorocitrate (Table IIIc). This either shows that the pool of GABA had a slow turnover, or that an unlabeled source, e.g. glutamine, substituted for glucose as a precursor for GABA. Fluorocitrate did not significantly affect neither the intracellular level nor the radiolabeling of aspartate even though the radiolabeling of glutamate was significantly reduced (Table IIIc).

DISCUSSION

The astrocytic cultures used in this experiment appeared free of neuronal contamination since no GABA or GABA labelling were detected. Likewise, the neuronal cultures seemed to be relatively free of astrocytic contamination since the labeling of glutamine from [1-^{14}C]glucose was so small, and since fluorocitrate, in contrast to what was the case in astrocytes, did not cause a rise in the formation of NH_3. This conclusion is based on the finding that glutamine synthetase (EC 6.3.1.2) is present in astrocytes and not in neurons (19).

The astrocytes cultured from cerebellum and frontal cortex did not differ much with respect to contents of amino acids, which is in accordance with previous findings (3). They did, however, differ with regard to release of metabolites to the extracellular fluid and in the response to fluorocitrate. The present study confirms the large release of citrate from cultured cortical astrocytes first demonstrated by Sonnewald et al. (6). Under similar conditions we show that cerebellar astrocytes release five times more citrate than the cortical astrocytes. It has previously been shown that citrate injected intracerebrally in vivo is rapidly taken up by astrocytes (10). It may be that astrocytes regulate the extracellular level of citrate, a function which appears important since increasing the extracellular concentration of citrate enhances neuronal excitability through chelation of divalent cations, as shown by Hornfeldt and Larson (20). Leprince et al. (21) reported on a factor of low molecular weight released by astrocytes, which was toxic to cultured cerebellar granule cells. However, incubating cerebellar neurons with 0.9 mM citrate for 24 hours (which is three times the concentration reached in the astrocytic culture medium after 24 hours of incubation), alone or together with glutamine (0.9 mM), did not cause significant neuronal death (B. Hassel, unpublished).

The cortical astrocytes differed from the cerebellar astrocytes in having a markedly lower release of metabolites (lactate + citrate + glutamine + alanine) relative to the consumption of glucose. This suggests that cortical astrocytes to a higher degree relied on oxidative metabolism of glucose. ^{13}C nuclear magnetic resonance (NMR) spectroscopy of cortical astrocytes incubated with [1-^{13}C]glucose showed that the only metabolites which were formed from glucose and released to the culture medium, were in fact lactate, citrate, glutamine and alanine (22). The consumed glucose which could not be accounted for by the above-mentioned metabolites in the present study was therefore in all likelihood converted to CO_2

Cerebellar and cortical astrocytes also exhibited differences in their response to fluorocitrate. The reduction in the intracellular levels of amino acids was more severe in the cortical than in the cerebellar astrocytes. Further, the per cent changes in the release of citrate and glutamine were greater in the cortical than in the cerebellar astrocytes. A likely explanation is that the higher level of citrate in the cerebellar astrocytes competed with the administered fluorocitrate for cellular uptake or binding to aconitase thus reducing the effect of fluorocitrate in these cells.

Astrocytes and neurons differed in their content of amino acids. Astrocytes contained higher levels of glutamine, taurine and glutathione than neurons. Taurine has previously been suggested as an astrocytic osmoregulator in the brain (4,23), and glutathione has been shown to have a predominantly glial localization in the intact brain (24). Although astrocytes contained more taurine and glutathione, both substances were present in cultured neurons in concentrations that probably cannot be explained by astrocytic contamination alone. Neurons contained higher levels of glutamate than the astrocytes did. Aspartate and GABA were especially high in cortical neurons, in agreement with earlier in vivo studies showing high aspartate-like immunoreactivity in GABAergic neurons (25). An additional finding in the present study was that increasing the extracellular concentration of glutamine led to higher intracellular levels of aspartate, but not of glutamate, in cortical neurons. This is relevant to previous studies showing that excitotoxic injury to cultured cortical neurons both during hypoxia and in glucose deprivation is dependent on the extracellular concentration of glutamine (26,27).

Both astrocytes and neurons showed signs of TCA cycle inhibition in the presence of fluorocitrate as judged from the increase in the release of citrate and the reduced

radiolabeling of intracellular amino acids. However, 100 μM fluorocitrate reduced the radiolabeling of glutamate by 50% in neurons as opposed to 90% in the astrocytes which reflects a greater degree of inhibition in the latter. The inhibitory effect of fluorocitrate on radiolabelling was also evident at lower concentrations in the astrocytes than in the neurons. Further, fluorocitrate reduced the intracellular levels of amino acids markedly more in astrocytes than in neurons. This is in agreement with previous in vivo findings which showed fluorocitrate to be selectively gliotoxic within a narrow dose range (9,10).

During fluorocitrate poisoning the release of lactate increased by 40% in neurons suggesting a compensatory increase in glycolysis. Astrocytes, on the other hand, reduced the release of lactate by approximately 30% during blockade of the TCA cycle with fluorocitrate. This finding suggests that formation of pyruvate and hence lactate from TCA cycle intermediates is a prominent process in astrocytes, and it is in agreement with two recent reports based on NMR spectroscopy of cultured astrocytes, which showed astrocytic release of lactate formed from TCA cycle intermediates (22,28). In this context it is of interest that cytosolic malic enzyme (EC 1.1.1.40), which converts malate to pyruvate, has been found in astrocytes, but not in neurons (5,29). The higher content of glutathione in astrocytes than in neurons may be relevant to this discussion, since reduction of oxidized glutathione requires NADPH (30) which is formed through the activity of malic enzyme.

Both in astrocytes and neurons the radiolabeling of aspartate was less affected by fluorocitrate than that of glutamate. This may reflect the fact that aspartate (in contrast to glutamate and glutamine) may be labeled from [^{14}C]glucose through metabolic pathways other than the straightforeward operation of the TCA cycle. In astrocytes carboxylation of [^{14}C]pyruvate to [^{14}C]oxaloacetate by pyruvate carboxylase (EC 6.4.1.1) is possible (31). In both cell types cleavage of [^{14}C]citrate to [^{14}C]oxaloacetate and acetyl-coA by ATP citrate lyase (EC 4.1.3.28) could take place (32).

In conclusion we wish to point out that cerebellar astrocytes released greater amounts of citrate, lactate and glutamine than the cortical astrocytes, whereas cortical astrocytes appeared more dependent on oxidative metabolism than the cerebellar astrocytes. Astrocytes contained higher levels of glutamine, taurine and glutathione than neurons, whereas neurons contained higher levels of glutamate. Cortical neurons were especially high in GABA and aspartate, and the level of aspartate increased with an increased extracellular concentration of glutamine. Fluorocitrate inhibited TCA cycle activity to a greater degree in astrocytes than in neurons, especially in the cortical astrocytes. During fluorocitrate poisoning the release of lactate increased in neurons suggesting a compensatory increase in glycolysis. In astrocytes, on the other hand, the release of lactate decreased, probably reflecting the glial formation of pyruvate from TCA cycle intermediates. The shift from oxidative metabolism to glycolysis seen in neurons, but not in astrocytes during fluorocitrate poisoning could contribute to the specificity of fluorocitrate as a gliotoxin.

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